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(54) Title: ANTHRAX TOXIN FUSION PROTEINS AND USES THEREOF		
(57) Abstract		
<p>The present invention provides a nucleic acid encoding a fusion protein comprising a nucleotide sequence encoding the anthrax protective antigen (PA) binding domain of the native anthrax lethal factor (LF) protein and a nucleotide sequence encoding an activity inducing domain of a second protein. Also provided is a nucleic acid encoding a fusion protein comprising a nucleotide sequence encoding the translocation domain and LF binding domain of the native anthrax PA protein and a nucleotide sequence encoding a ligand domain which specifically binds a cellular target. Proteins encoded by the nucleic acid of the invention are also provided, as well as a method for delivering an activity to a cell using such fusion proteins. The invention also provides proteins including an anthrax protective antigen which has been mutated to replace the trypsin cleavage site with residues recognized specifically by the HIV-1 protease.</p>		

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ANTHRAX TOXIN FUSION PROTEINS AND USES THEREOF

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This application is in a continuation in part application of Serial No. 08/021,601 filed February 12, 1993.

BACKGROUND OF THE INVENTION

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The targeting of cytotoxic or other moieties to specific cell types has been proposed as a method of treating diseases such as cancer. Various toxins including *Diphtheria* toxin and *Pseudomonas* exotoxin A have been suggested as potential candidate toxins for this type of treatment. A difficulty of such methods has been the inability to selectively target specific cell types for the delivery of toxins or other active moieties.

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One method of targeting specific cells has been to make fusion proteins of a toxin and a single chain antibody. A single-chain antibody (sFv) consists of an antibody light chain variable domain (V_L) and heavy chain variable domain (V_H), connected by a short peptide linker which allows the structure to assume a conformation capable of binding to antigen. In a diagnostic or therapeutic setting, the use of an sFv may offer attractive advantages over the use of a monoclonal antibody (MoAb). Such advantages include more rapid tumor penetration with concomitantly low retention in non-targeted organs (Yokota et al. *Cancer Res* 52:3402, 1992), extremely rapid plasma and whole body clearance (resulting in high tumor to normal tissue partitioning) in the course of imaging studies (Colcher et al. *Natl. Cancer Inst.* 82: 1191, 1990; Milenic et al. *Cancer Res.* 51:6363, 1991), and relatively low cost of production and ease of manipulation at the genetic level (Huston et al. *Methods Enzymol.* 203:46, 1991; Johnson, S. and Bird, R. E. *Methods Enzymol.* 203:88, 1991). In addition, sFv-toxin fusion proteins have been shown to exhibit enhanced anti-tumor activity in comparison with conventional chemically cross-linked conjugates (Chaudhary et

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al. *Nature* 339:394, 1989; Batra et al. *Cell. Biol.* 11:2200-2295, 1991). Among the first sFv to be generated were molecules capable of binding haptens (Bird et al. *Science* 242:423, 1988; Huston et al. *Proc. Natl. Acad. Sci. USA* 85:5879, 1988), cell-surface receptors (Chaudhary et al., 1989), and tumor antigens (Chaudhary et al. *Proc. Natl. Acad. Sci. USA* 87:1066, 1990; Colcher et al., 1990).

The gene encoding an sFv can be assembled in one of two ways: (i) by *de novo* construction from chemically synthesized overlapping oligonucleotides, or (ii) by polymerase chain reaction (PCR)-based cloning of V_L and V_H genes from hybridoma cDNA. The main disadvantages of the first approach are the considerable expense involved in oligonucleotide synthesis, and the fact that the sequence of V_L and V_H must be known before gene assembly is possible. Consequently, the majority of the sFv reported to date were generated by cloning from hybridoma cDNA; nevertheless, this approach also has inherent disadvantages, because it requires availability of the parent hybridoma or myeloma cell line, and problems are often encountered when attempting to retrieve the correct V region genes from heterologous cDNA. For example, hybridomas in which the immortalizing fusion partner is derived from MOPC-21 may express a V_L kappa transcript which is aberrantly rearranged at the VJ recombination site, and which therefore encodes a non-functional light chain (Cabilly & Riggs, 1985; Carroll et al., 1988). Cellular levels of this transcript may exceed that generated from the productive V_L gene, so that a large proportion of the product on PCR amplification of hybridoma cDNA will not encode a functional light chain. A second disadvantage of the PCR-based method, frequently encountered by the inventors, is the variable success of recovering V_H genes using the conditions so far reported in the literature, presumably because the number of mismatches between primers and the target sequence destabilizes the hybrid to an extent which inhibits PCR amplification.

Thus, methods of targeting toxins to specific cells using single-chain antibodies methods have been difficult to

practice because of the difficulties in obtaining single chain antibodies and other targeting moieties. Also, none of the proposed treatment methods has been fully successful, because of the need to fuse the toxin to the targeting moiety, thus
5 disrupting either the toxin function or the targeting function. Thus, a need exists for a means to target molecules having a desired activity to a specific cell population.

Bacterial and plant protein toxins have evolved novel and efficient strategies for penetrating to the cytosol
10 of mammalian cells, and this ability has been exploited to develop anti-tumor and anti-HIV cytotoxic agents. Examples include ricin and *Pseudomonas* exotoxin A (PE) chimeric toxins and immunotoxins.

Pseudomonas exotoxin A (PE) is a toxin for which a
15 detailed analysis of functional domains exists. The sequence is deposited with GenBank. Structural determination by X-ray diffraction, expression of deleted proteins, and extensive mutagenesis studies have defined three functional domains in PE: a receptor-binding domain (residues 1-252 and 365-399)
20 designated Ia and Ib, a central translocation domain (amino acids 253-364, domain II), and a carboxyl-terminal enzymatic domain (amino acids 400-613, domain III). Domain III catalyzes the ADP-ribosylation of elongation factor 2 (EF-2), which results in inhibition of protein synthesis and cell
25 death. Recently it was also found that an extreme carboxyl terminal sequence is essential for toxicity (Chaudhary et al. *Proc. Natl. Acad. Sci. U.S.A.* 87:308-312, 1990; Seetharam et al. *J. Biol. Chem.* 266:17376-17381, 1991). Since this sequence is similar to the sequence that specifies retention
30 of proteins in the endoplasmic reticulum (ER) (Munro, S. and Pelham, H.R.B. *Cell* 48:899-907, 1987), it was suggested that PE must pass through the ER to gain access to the cytosol. Detailed knowledge of the structure of PE has facilitated use of domains II, Ib, and III (together designated PE40) in
35 hybrid toxins and immunotoxins.

Bacillus anthracis produces three proteins which when combined appropriately form two potent toxins, collectively designated anthrax toxin. Protective antigen

(PA, 82,684 Da (Dalton) (SEQ ID NOS: 3 and 4)) and edema factor (EF, 89,840 Da) combine to form edema toxin (ET), while PA and lethal factor (LF, 90,237 Da (SEQ ID NOS: 1 and 2)) combine to form lethal toxin (LT) (Leppla, S.H. Alouf, J.E. and Freer, J. H., eds. *Academic Press, London* 277-302, 1991). ET and LT each conform to the AB toxin model, with PA providing the target cell binding (B) function and EF or LF acting as the effector or catalytic (A) moieties. A unique feature of these toxins is that LF and EF have no toxicity in the absence of PA, apparently because they cannot gain access to the cytosol of eukaryotic cells.

The genes for each of the three anthrax toxin components have been cloned and sequenced (Leppla, 1991). This showed that LF and EF have extensive homology in amino acid residues 1-300. Since LF and EF compete for binding to PA63, it is highly likely that these amino-terminal regions are responsible for binding to PA63. Direct evidence for this was provided in a recent mutagenesis study (Quinn et al. *J. Biol. Chem.* 266:20124-20130, 1991); all mutations made within amino acid residues 1-210 of LF led to decreased binding to PA63. The same study also suggested that the putative catalytic domain of LF included residues 491-776 (Quinn et al., 1991). In contrast, the location of functional domains within the PA63 polypeptide is not obvious from inspection of the deduced amino acid sequence. However, studies with monoclonal antibodies and protease fragments (Leppla, 1991) and subsequent mutagenesis studies (Singh et al. *J. Biol. Chem.* 266:15493-15497, 1991) showed that residues at and near the carboxyl terminus of PA are involved in binding to receptor.

PA is capable of binding to the surface of many types of cells. After PA binds to a specific receptor (Leppla, 1991) on the surface of susceptible cells, it is cleaved at a single site by a cell surface protease, probably furin, to produce an amino-terminal 19-kDa fragment that is released from the receptor/PA complex (Singh et al. *J. Biol. Chem.* 264:19103-19107, 1989). Removal of this fragment from PA exposes a high-affinity binding site for LF and EF on the

receptor-bound 63-kDa carboxyl-terminal fragment (PA63). The complex of PA63 and LF or EF enters cells and probably passes through acidified endosomes to reach the cytosol.

Cleavage of PA occurs after residues 164-167, Arg-Lys-Lys-Arg. This site is also susceptible to cleavage by trypsin and can be referred to as the trypsin cleavage site. Only after cleavage is PA able to bind either EF or LF to form either ET or LT.

Prior work had shown that the carboxyl terminal PA fragment (PA63) can form ion conductive channels in artificial lipid membranes (Blaustein et al. *Proc. Natl. Acad. Sci. U.S.A.* 86:2209-2213, 1989; Koehler, T. M. and Collier, R.J. *Mol. Microbiol.* 5:1501-1506, 1991), and that LF bound to PA63 on cell surface receptors can be artificially translocated across the plasma membrane to the cytosol by acidification of the culture medium (Friedlander, A. M. *J. Biol. Chem.* 261:7123-7126, 1986). Furthermore, drugs that block endosome acidification protect cells from LF (Gordon et al. *J. Biol. Chem.* 264:14792-14796, 1989; Friedlander, 1986; Gordon et al. *Infect. Immun.* 56:1066-1069, 1988). The mechanisms by which EF is internalized have been studied in cultured cells by measuring the increases in cAMP concentrations induced by PA and EF (Leppla, S. H. *Proc. Natl. Acad. Sci. U.S.A.* 79:3162-3166, 1982; Gordon et al., 1989). However, because assays of cAMP are relatively expensive and not highly precise, this is not a convenient method of analysis. Internalization of LF has been analyzed only in mouse and rat macrophages, because these are the only cell types lysed by the lethal toxin.

SUMMARY OF THE INVENTION

The present invention provides a nucleic acid encoding a fusion protein comprising a nucleotide sequence encoding the PA binding domain of the native LF protein and a nucleotide sequence encoding an activity inducing domain of a second protein. Also provided is a nucleic acid encoding a fusion protein comprising a nucleotide sequence encoding the translocation domain and LF binding domain of the native PA protein and a nucleotide sequence encoding a ligand domain

which specifically binds a cellular target. Proteins encoded by the nucleic acid of the invention, vectors comprising the nucleic acids and hosts capable of expressing the protein encoded by the nucleic acids are also provided.

5 A composition comprising the PA binding domain of the native LF protein chemically attached to an activity inducing moiety is further provided.

A method for delivering an activity to a cell is provided. The steps of the method include administering to
10 the cell (a) a protein comprising the translocation domain and the LF binding domain of the native PA protein and a ligand domain and (b) a product comprising the PA binding domain of the native LF protein and a non-LF activity inducing moiety, whereby the product administered in step (b) is internalized
15 into the cell and performs the activity within the cell.

Characteristics unique to anthrax toxin are exploited to make novel cell-specific cytotoxins. A site in the PA protein of the toxin which must be proteolytically cleaved for the activity-inducing moiety of the toxin to enter
20 the cell is replaced by the consensus sequence recognized by a specific protease. Thus, the toxin will only act on cells infected with intracellular pathogens which make that specific protease.

25 BRIEF DESCRIPTION OF THE DRAWINGS

Figure 1 is a graph of the percent to which mutant proteins are cleaved by purified HIV-1 protease. The mutant proteins include protective antigen (PA) mutated to include the HIV-1 protease cleavage site in place of the natural
30 trypsin cleavage site.

DESCRIPTION OF THE PREFERRED EMBODIMENT

Nucleic Acids

Lethal Factor (LF)

35 The present invention provides an isolated nucleic acid encoding a fusion protein comprising a nucleotide sequence encoding the PA binding domain of the native LF protein and a nucleotide sequence encoding an activity

inducing domain of a second protein. The LF gene and native LF protein are shown in SEQ ID NO: 1 and 2, respectively. The PA gene and native PA protein are shown in SEQ ID NO: 3 and 4, respectively.

5 The second protein can be a toxin, for example *Pseudomonas* exotoxin A (PE), the A chain of *Diphtheria* toxin or shiga toxin. The activity inducing domains of numerous other known toxins can be included in the fusion protein encoded by the presently claimed nucleic acid. The activity
10 inducing domain need not be a toxin, but can have other activities, including but not limited to stimulating or reducing growth, selectively inhibiting DNA replication, providing a desired gene, providing enzymatic activity or providing a source of radiation. In any case, the fusion
15 proteins encoded by the nucleic acids of the present invention must be capable of being internalized and capable of expressing the specified activity in a cell. A given LF fusion protein of the present invention can be tested for its ability to be internalized and to express the desired activity
20 using methods as described herein, particularly in Examples 1 and 2.

 An example of a nucleic acid of the invention comprises the nucleotide sequence defined in the Sequence Listing as SEQ ID NO: 5. This nucleic acid encodes a fusion
25 of LF residues 1-254 with the two-residue linker "TR" and PE residues 401-602 (SEQ ID NO: 6). The protein includes a Met-Val-Pro- sequence at the beginning of the LF sequence. Means for obtaining this fusion protein are further described below and in Example 1.

30 A further example of a nucleic acid of this invention comprises the nucleotide sequence defined in the Sequence Listing as SEQ ID NO: 7. This nucleic acid encodes a fusion of LF residues 1-254 with the two-residue linker "TR" and PE residues 398-613. (SEQ ID NO: 8) The junction point
35 containing the "TR" is the sequence LTRA and the Met-Val-Pro- is also present. This fusion protein and methods for obtaining it are further described below and in Example 2.

Another example of the nucleic acid of the present invention comprises the nucleotide sequence defined in the Sequence Listing as SEQ ID NO: 9. This nucleic acid encodes a fusion of LF residues 1-254 with the two residue linker and PE residues 362-613. (SEQ ID NO: 10) This fusion protein is further described in Example 1.

Alternatively, the nucleic acid can include the entire coding sequence for the LF protein fused to a non-LF activity inducing domain. Other LF fusion proteins of various sizes and methods of making and testing them for the desired activity are also provided herein, particularly in Examples 1 and 2.

Protective Antigen (PA)

Also provided is an isolated nucleic acid encoding a fusion protein comprising a nucleotide sequence encoding the translocation domain and LF binding domain of the native PA protein and a nucleotide sequence encoding a ligand domain which specifically binds a cellular target.

An example of a nucleic acid of this invention comprises the nucleotide sequence defined in the Sequence Listing as SEQ ID NO:11. This nucleic acid encodes a fusion of PA residues 1-725 and human CD4 residues 1-178, the portion which binds to gp120 exposed on HIV-1 infected cells (SEQ ID NO:12). This fusion protein and methods for obtaining and testing fusion proteins are further described below and in Examples 3, 4 and 5.

The PA fusion protein encoding nucleic acid provided can encode any ligand domain that specifically binds a cellular target, e.g. a cell surface receptor, an antigen expressed on the cell surface, etc. For example, the nucleic acid can encode a ligand domain that specifically binds to an HIV protein expressed on the surface of an HIV-infected cell. Such a ligand domain can be a single chain antibody which is expressed as a fusion protein as provided above and in Examples 3, 4 and 5. Alternatively, the nucleic acid can encode, for example, a ligand domain that is a growth factor, as provided in Example 3.

Although the PA encoding sequence of the nucleic acid encoding the PA fusion proteins of this invention need only include the nucleotide sequence encoding the translocation domain and LF binding domain of the native PA protein, the nucleic acid can further comprise the nucleotide sequence encoding the remainder of the native PA protein. Any sequences to be included beyond those required, can be determined based on routine considerations such as ease of manipulation of the nucleic acid, ease of expression of the product in the host, and any effect on translocation/internalization as taught in the examples.

Proteins

Proteins encoded by the nucleic acids of the present invention are also provided.

LF Fusion Proteins

The present invention provides LF fusion proteins encoded by the nucleic acids of the invention as described above and in the examples. Specifically, fusions of the LF gene with domains II, Ib, and III of PE can be made by recombinant methods to produce in-frame translational fusions. Recombinant genes (e.g., SEQ ID NOS: 5, 7 and 9) were expressed in *Escherichia coli* (*E. coli*), and the purified proteins were tested for activity on cultured cells as provided in Examples 1 and 2. Certain fusion proteins are efficiently internalized via the PA receptor to the cytosol. These examples demonstrate that this system can be used to deliver many different polypeptides into targeted cells.

Although specific examples of these proteins are provided, given the present teachings regarding the preparation of LF fusion proteins, other embodiments having other activity inducing domains can be practiced using routine skill.

Using current methods of genetic manipulation, a variety of other activity inducing moieties (e.g., polypeptides) can be translated as fusion proteins with LF which in turn can be internalized by cells when administered with PA or PA fusion proteins. Fusion proteins generated by

this method can be screened for the desired activity using the methods set forth in the Examples and by various routine procedures. Based on the data presented here, the present invention provides a highly effective system for delivery of an activity inducing moiety into cells.

PA fusion proteins

The present invention provides PA fusion proteins encoded by the nucleic acids of the invention. Specifically fusions of PA with single chain antibodies and CD4 are provided.

Using current methods of genetic manipulation, a variety of other ligand domains (e.g., polypeptides) can be translated as fusion proteins with PA which in turn can specifically target cells and facilitate internalization LF or LF fusion proteins. Based on the data presented here, the present invention provides a highly effective system for delivery of an activity inducing moiety into a particular type or class of cells.

Although specific examples of these proteins are provided, given the present teachings regarding the preparation of PA fusion proteins, other embodiments having other ligand domains can be practiced using routine skill. The fusion proteins generated can be screened for the desired specificity and activity utilizing the methods set forth in the example and by various routine procedures. In any case, the PA fusion proteins encoded by the nucleic acids of the present invention must be able to specifically bind the selected target cell, bind LF or LF fusions or conjugates and internalize the LF fusion/conjugate.

Conjugates

A composition comprising the PA binding domain of the native LF protein chemically attached to an activity inducing moiety is provided. Such an activity inducing moiety is an activity not present on native LF. The composition can comprise an activity inducing moiety that is, for example, a polypeptide, a radioisotope, an antisense nucleic acid or a nucleic acid encoding a desired gene product.

Using current methods of chemical manipulation, a variety of other moieties (e.g., polypeptides, nucleic acids, radioisotopes, etc.) can be chemically attached to LF and can be internalized into cells and can express their activity when administered with PA or PA fusion proteins. The compounds can be tested for the desired activity and internalization following the methods set forth in the Examples. For example, the present invention provides an LF protein fragment 1-254 (LF1-254) with a cysteine residue added at the end of LF1-254 (LF1-254Cys). Since there are no other cysteines in LF, this single cysteine provides a convenient attachment point through which to chemically conjugate other proteins or non-protein moieties.

Vectors and Hosts

A vector comprising the nucleic acids of the present invention is also provided. The vectors of the invention can be in a host capable of expressing the protein encoded by the nucleic acid.

To express the proteins and conjugates of the present invention, the nucleic acids can be operably linked to signals that direct gene expression. A nucleic acid is "operably linked" when it is placed into a functional relationship with another nucleic acid sequence. For instance, a promoter or enhancer is operably linked to a coding sequence if it affects the transcription of the sequence. Generally, operably linked means that the nucleic acid sequences being linked are contiguous and, where necessary to join two protein coding regions, contiguous and in reading frame.

The gene encoding a protein of the invention can be inserted into an "expression vector", "cloning vector", or "vector," terms which usually refer to plasmids or other nucleic acid molecules that are able to replicate in a chosen host cell. Expression vectors can replicate autonomously, or they can replicate by being inserted into the genome of the host cell. Vectors that replicate autonomously will have an origin of replication or autonomous replicating sequence (ARS) that is functional in the chosen host cell(s). Often, it is

desirable for a vector to be usable in more than one host cell, e.g., in *E. coli* for cloning and construction, and in a mammalian cell for expression.

The particular vector used to transport the genetic information into the cell is also not particularly critical. Any of the conventional vectors used for expression of recombinant proteins in prokaryotic or eukaryotic cells can be used.

The expression vectors typically have a transcription unit or expression cassette that contains all the elements required for the expression of the DNA encoding a protein of the invention in the host cells. A typical expression cassette contains a promoter operably linked to the DNA sequence encoding the protein, and signals required for efficient polyadenylation of the transcript. The promoter is preferably positioned about the same distance from the heterologous transcription start site as it is from the transcription start site in its natural setting. As is known in the art, however, some variation in this distance can be accommodated without loss of promoter function.

The DNA sequence encoding the protein of the invention can be linked to a cleavable signal peptide sequence to promote secretion of the encoded protein by the transformed cell. Additional elements of the vector can include, for example, selectable markers and enhancers. Selectable markers, e.g., tetracycline resistance or hygromycin resistance, permit detection and/or selection of those cells transformed with the desired DNA sequences (see, e.g., U.S. Patent 4,704,362).

Enhancer elements can stimulate transcription up to 1,000 fold from linked homologous or heterologous promoters. Many enhancer elements derived from viruses have a broad host range and are active in a variety of tissues. For example, the SV40 early gene enhancer is suitable for many cell types. Other enhancer/promoter combinations that are suitable for the present invention include those derived from polyoma virus, human or murine cytomegalovirus, the long terminal repeat from various retroviruses such as murine leukemia virus, murine or

Rous sarcoma virus, and HIV. See, *Enhancers and Eukaryotic Expression*, Cold Spring Harbor Pres, Cold Spring Harbor, N.Y. 1983, which is incorporated herein by reference.

In addition to a promoter sequence, the expression cassette should also contain a transcription termination region downstream of the structural gene to provide for efficient termination. The termination region can be obtained from the same gene as the promoter sequence or can be obtained from a different gene.

For more efficient translation in mammalian cells of the mRNA encoded by the structural gene, polyadenylation sequences are also commonly added to the vector construct. Two distinct sequence elements are required for accurate and efficient polyadenylation: GU or U rich sequences located downstream from the polyadenylation site and a highly conserved sequence of six nucleotides, AAUAAA, located 11-30 nucleotides upstream. Termination and polyadenylation signals that are suitable for the present invention include those derived from SV40, or a partial genomic copy of a gene already resident on the expression vector.

The vectors containing the gene encoding the protein of the invention are transformed into host cells for expression. "Transformation" refers to the introduction of vectors containing the nucleic acids of interest directly into host cells by well known methods. The particular procedure used to introduce the genetic material into the host cell for expression of the protein is not particularly critical. Any of the well known procedures for introducing foreign nucleotide sequences into host cells can be used. It is only necessary that the particular procedure utilized be capable of successfully introducing at least one gene into the host cell which is capable of expressing the gene.

Transformation methods, which vary depending on the type of host cell, include electroporation; transfection employing calcium chloride, rubidium chloride calcium phosphate, DEAE-dextran, or other substances; microprojectile bombardment; lipofection; infection (where the vector is an infectious agent); and other methods. See, generally,

Sambrook et al., (1989) *supra*, and *Current Protocols in Molecular Biology, supra*. Reference to cells into which the nucleic acids described above have been introduced is meant to also include the progeny of such cells.

5 There are numerous prokaryotic expression systems known to one of ordinary skill in the art useful for the expression of the antigen. *E. coli* is commonly used, and other microbial hosts suitable for use include *bacilli*, such as *Bacillus subtilis*, and other enterobacteriaceae, such as
10 *Salmonella*, *Serratia*, and various *Pseudomonas* species. One can make expression vectors for use in these prokaryotic hosts; the vectors will typically contain expression control sequences compatible with the host cell (e.g., an origin of replication, a promoter). Any number of a variety of well-
15 known promoters can be used, such as the lactose promoter system, a tryptophan (Trp) promoter system, a beta-lactamase promoter system, or a promoter from phage lambda. The promoters will typically control expression, optionally with an operator sequence, and have ribosome binding site
20 sequences, for example, for initiating and completing transcription and translation. If necessary, an amino terminal methionine can be provided by insertion of a Met codon 5' and in-frame with the codons for the protein. Also, the carboxy-terminal end of the protein can be removed using
25 standard oligonucleotide mutagenesis procedures, if desired.

Host bacterial cells can be chosen that are mutated to be reduced in or free of proteases, so that the proteins produced are not degraded. For *Bacillus* expression systems in which the proteins are secreted into the culture medium,
30 strains are available that are deficient in secreted proteases.

Mammalian cell lines can also be used as host cells for the expression of polypeptides of the invention. Propagation of mammalian cells in culture is *per se* well
35 known. See, *Tissue Culture*, Academic Press, Kruse and Patterson, ed. (1973). Host cell lines may also include such organisms as bacteria (e.g., *E. coli* or *B. subtilis*), yeast, filamentous fungi, plant cells, or insect cells, among others.

Purification of Protein

After standard transfection or transformation methods are used to produce prokaryotic, mammalian, yeast, or insect cell lines that express large quantities of the protein of the invention, the protein is then purified using standard techniques which are known in the art. See, e.g., Colley et al. (1989) *J. Biol. Chem.* 64: 17619-17622; and *Methods in Enzymology*, "Guide to Protein Purification", M. Deutscher, ed. Vol. 182 (1990).

Standard procedures of the art that can be used to purify proteins of the invention include ammonium sulfate precipitation, affinity and fraction column chromatography, gel electrophoresis and the like. See, generally, Scopes, R., *Protein Purification*, Springer-Verlag, New York (1982), and U.S. Pat. No. 4,512,922 disclosing general methods for purifying protein from recombinantly engineered bacteria.

If the expression system causes the protein of the invention to be secreted from the cells, the recombinant cells are grown and the protein is expressed, after which the culture medium is harvested for purification of the secreted protein. The medium is typically clarified by centrifugation or filtration to remove cells and cell debris and the proteins can be concentrated by adsorption to any suitable resin such as, for example, CDP-Sepharose, asialoproteothrombin-Sepharose 4B, or Q Sepharose, or by use of ammonium sulfate fractionation, polyethylene glycol precipitation, or by ultrafiltration. Other means known in the art are equally suitable. Further purification of the protein can be accomplished by standard techniques, for example, affinity chromatography, ion exchange chromatography, sizing chromatography, or other protein purification techniques used to obtain homogeneity. The purified proteins are then used to produce pharmaceutical compositions, as described below.

Alternatively, vectors can be employed that express the protein intracellularly, rather than secreting the protein from the cells. In these cases, the cells are harvested, disrupted, and the protein is purified from the cellular extract, e.g., by standard methods. If the cell line has a

cell wall, then initial extraction in a low salt buffer may allow the protein to pellet with the cell wall fraction. The protein can be eluted from the cell wall with high salt concentrations and dialyzed. If the cell line glycosylates the protein, then the purified glycoprotein may be enhanced by using a Con A column. Anion exchange columns (MonoQ, Pharmacia) and gel filtration columns may be used to further purify the protein. A highly purified preparation can be achieved at the expense of activity by denaturing preparative polyacrylamide gel electrophoresis.

Protein analogs can be produced in multiple conformational forms which are detectable under nonreducing chromatographic conditions. Removal of those species having a low specific activity is desirable and is achieved by a variety of chromatographic techniques including anion exchange or size exclusion chromatography.

Recombinant analogs can be concentrated by pressure dialysis and buffer exchanged directly into volatile buffers (e.g., N-ethylmorpholine (NEM), ammonium bicarbonate, ammonium acetate, and pyridine acetate). In addition, samples can be directly freeze-dried from such volatile buffers resulting in a stable protein powder devoid of salt and detergents. In addition, freeze-dried samples of recombinant analogs can be efficiently resolubilized before use in buffers compatible with infusion (e.g., phosphate buffered saline). Other suitable buffers might include hydrochloride, hydrobromide, sulphate acetate, benzoate, malate, citrate, glycine, glutamate, and aspartate.

Specific Embodiments

Toxins Modified to Contain Intracellular Pathogen Protease Recognition sites

One aspect of the invention exploits the fact that PA and other toxins must be proteolytically cleaved in order to acquire activity, in conjunction with the fact that some cells infected with an intracellular pathogen possess an active protease that has a relatively narrow substrate specificity (for example, HIV-infected cells). The protease

site found in the native toxin is replaced with an intracellular pathogen specific protease site. Thus, the protease in cells that are infected by the intracellular pathogen cleaves the modified toxin, which then becomes active and kills the cell.

Intracellular pathogens that can be targeted by the products and methods of the present invention include any pathogen that produces a protease having a specific recognition site. Such pathogens can include prokaryotes (including rickettsia, *Mycobacterium tuberculosis*, etc.), mycoplasma, eukaryotic pathogens (e.g. pathogenic fungi, etc.), and viruses. One example of an intracellular pathogen that produces a specific protease is human immunodeficiency virus (HIV). The HIV-1 protease cleaves viral polyproteins to generate functional structural proteins as well as the reverse transcriptase and the protease itself. HIV-1 replication and viral infectivity are absolutely dependent on the action of the HIV-1 protease.

An intracellular pathogen specific protease site can be introduced into any natural or recombinant toxin for which proteolytic cleavage is required for toxicity. For example, one can replace the anthrax PA trypsin cleavage site (R164-167) of PA with the HIV-1 protease site. Alternatively, the diphtheria toxin disulfide loop sequence (see O'Hare, et al. FEBS 273 (1, 2): 200-204 (Oct. 1990)) can be replaced with the HIV-1 protease cleavage site in order to obtain a toxin specific to HIV-1 infected cells. Similarly, the normally occurring diphtheria toxin sequence at residues 191-194 (Williams, et al. *J. Biol. Chem.* 265(33): 20673-20677 (1990)) can be replaced by an intracellular pathogen specific protease site such as the HIV-1 protease cleavage sequence. The DAB486-IL-2 fusion toxin of Williams and the improved DAB389-IL-2 toxin are effective on HIV-1 infected cells, which express high levels of the IL-2 receptor. Williams, *J. Biol. Chem.* 265:20673. Addition of the HIV-1 protease cleavage site would provide a further degree of specificity. Similarly the botulinum toxin C2 toxin is like the anthrax toxin in requiring a cleavage within a native protein subunit (see

Ohishi and Yanagimoto, *Infection and Immunity* 60(11): 4648-4655 (Nov. 1992)), so it too can be made specific for cells infected by an intracellular pathogen such as HIV-1.

In one embodiment of the invention, the protease site of PA is replaced by the site recognized by the HIV-1 protease. The cellular protease that cleaves PA absolutely requires the presence of the Arg 164 and Arg 167 residues, because replacement of either residue yields a PA molecule which is not cleaved after binding to the cell surface.

However, any PA substitution mutant which retains at least one Arg or Lys residue within residues 164-167 can be activated by treatment with trypsin. Because the PA63 fragments produced by trypsin digestion have a variety of different amino terminal residues, it is clear that there is not a strict constraint on the identity of the terminal residues. Klimpel, et al., *Proc. Natl. Acad. Sci.* 89:10277-10281 (1992).

Replacement of residues 164-167 of PA with residues that match the HIV-1 protease recognition site can render exogenously added PA inactive on cells which do not possess the HIV-1 protease. However, those cells that do express the HIV-1 protease (i.e., cells infected with HIV-1 or cells engineered to produce the protease) would cleave and thereby activate the mutant PA. The activated PA proteins can then bind and internalize cytotoxic fusion proteins, such as LF-PE, added exogenously.

Based on extensive studies of the substrate specificity of the protease, several PA variants were designed and produced which relate to the invention. These are shown below, with the residues underlined between which the cleavage occurred. PA proteins which have been mutated to replace R164-167 with an amino acid sequence recognized by the HIV-1 protease are referred to as "PAHIV."

PAHIV#1	QVSONY <u>P</u> IVQNI
PAHIV#2	NTATIM <u>M</u> QRGNF
PAHIV#3	TVSFNF <u>P</u> QITLW
PAHIV#4	GGSAFNF <u>P</u> IVMGG

The mutant proteins PAHIV#(1-4) were cleaved correctly by the HIV-1 protease.

Table 1 shows the amino acids and their corresponding abbreviations and symbols.

Table 1

A	Ala	Alanine	M	Met	Methionine
C	Cys	Cysteine	N	Asn	Asparagine
D	Asp	Aspartic acid	P	Pro	Proline
E	Glu	Glutamic acid	Q	Gln	Glutamine
F	Phe	Phenylalanine	R	Arg	Arginine
G	Gly	Glycine	S	Ser	Serine
H	His	Histidine	T	Thr	Threonine
I	Ile	Isoleucine	V	Val	Valine
K	Lys	Lysine	W	Trp	Tryptophan
L	Leu	Leucine	Y	Tyr	Tyrosine

Preferably, the mutations at R164-167 of PA are accomplished by cassette mutagenesis, although other methods are feasible as discussed below. In summary, three pieces of DNA are joined together. The first piece has vector sequences and encodes the "front half" (5' end of the gene) of PA protein, the second is a short piece of DNA (a cassette) and encodes a small middle piece of PA protein and the third encodes the "back half" (3' end of the gene) of PA. The cassette contains codons for the amino acids that are required to complete the cleavage site for the intracellular pathogen protease. This method was used to make mutants in the plasmid pYS5 although other plasmids could be employed.

Alternatively, the mutations can be accomplished by use of the polymerase chain reaction (PCR) and other methods as discussed below. PCR duplicates a segment of DNA many times, resulting in an amplification of that segment. The reaction produces enough of the segment of DNA so that it can be modified with restriction enzymes and cloned. During the reaction a synthetic oligonucleotide primer is used to start the duplication of the target DNA segment. Each synthetic

primer can be designed to introduce novel DNA sequences into the DNA molecule, or to change existing DNA sequences.

Modification of Toxins to Broaden or Alter Target Cell

5 Specificity

Another aspect of the invention involves compounds and methods for broadening or changing the range of cell types against which a toxin is effective. For example, the lethal anthrax toxin, PA+LF, is acutely toxic to mouse macrophage
10 cells, apparently due to the specific expression in these cells of a target for the catalytic activity of LF. Other cell types are not affected by LF. However, in the present invention, LF is used to construct cytotoxins having broad cell specificity.

15 A detailed analysis of the domains of LF identified the amino-terminal 254 amino acids as the region that binds to PA63. Fusion proteins containing residues 1-254 of LF and the ADP-ribosylation domain of *Pseudomonas* exotoxin A (PE) were designed according to the invention. These fusion proteins
20 are highly toxic to cultured cells, but only when PA is administered simultaneously.

Synthesis of Genes that Encode Proteins of the Invention

Genes that encode toxins having altered protease recognition sites or fusion proteins having a binding domain
25 from one protein and an activity inducing domain of a second protein can be synthesized by methods known to those skilled in the art. As an example of techniques that can be utilized, the synthesis of genes encoding modified anthrax toxin subunits LF and PA are now described.

30 The DNA sequences for native PA and LF are known. Knowledge of these DNA sequences facilitates the preparation of genes and can be used as a starting point to construct DNA molecules that encode mutants of PA and/or LF. The protein mutants of the invention are soluble and include internal
35 amino acid substitutions. Furthermore, these mutants are purified from, or secreted from, cells that have been transfected or transformed with plasmids containing genes which encode these proteins. Methods for making

modifications, such as amino acid substitutions, deletions, or the addition of signal sequences to cloned genes are known. Specific methods used herein are described below.

The gene for PA or LF can be prepared by several methods. Genomic and cDNA libraries are commercially available. Oligonucleotide probes, specific to the desired gene, can be synthesized using the known gene sequence. Methods for screening genomic and cDNA libraries with oligonucleotide probes are known. A genomic or cDNA clone can provide the necessary starting material to construct an expression plasmid for the desired protein using known methods.

A protein encoding DNA fragment can be cloned by taking advantage of restriction endonuclease sites which have been identified in regions which flank or are internal to the gene. See Sambrook, et al., *Molecular Cloning: A Laboratory Manual* 2d.ed. Cold Spring Harbor Laboratory Press (1989), "Sambrook" hereinafter.

Genes encoding the desired protein can be made from wild-type genes constructed using the gene encoding the full length protein. One method for producing wild-type genes for subsequent mutation combines the use of synthetic oligonucleotide primers with polymerase extension on a mRNA or DNA template. This PCR method amplifies the desired nucleotide sequence. U.S. Patents 4,683,195 and 4,683,202 describe this method. Restriction endonuclease sites can be incorporated into the primers. Genes amplified by PCR can be purified from agarose gels and cloned into an appropriate vector. Alterations in the natural gene sequence can be introduced by techniques such as *in vitro* mutagenesis and PCR using primers that have been designed to incorporate appropriate mutations.

The proteins described herein can be expressed intracellularly and purified, or can be secreted when expressed in cell culture. If desired, secretion can be obtained by the use of the native signal sequence of the gene. Alternatively, genes encoding the proteins of the invention can be ligated in proper reading frame to a signal sequence

other than that corresponding to the native gene. Though the PA recombinant proteins of the invention are typically expressed in *B. anthracis*, they can be expressed in other hosts, such as *E. coli*.

5 The proteins of this invention are described by their amino acid sequences and by their nucleotide sequence, it being understood that the proteins include their biological equivalents such that this invention includes minor or inadvertent substitutions and deletions of amino acids that
10 have substantially little impact on the biological properties of the analogs. In some circumstances it may be feasible to substitute rare or non-naturally occurring amino acids for one or more of the twenty common amino acids listed in Table 2. Examples include ornithine and acetylated or hydroxylated
15 forms. See generally Stryer, L., *Biochemistry* 3d ed. (1988).

 Alternative nucleotide sequences can be used to express analogs in various host cells. Furthermore, due to the degeneracy of the genetic code, equivalent codons can be substituted to encode the same polypeptide sequence.

20 Additionally, sequences (nucleotide and amino acid) with substantial identity to those of the invention are also included. Identity in this sense means the same identity (of base pair or amino acid) and order (of base pairs or amino acids). Substantial identity includes entities that are
25 greater than 80% identical. Preferably, substantial identity refers to greater than 90% identity. More preferably, it refers to greater than 95% identity.

Mutagenesis

30 Mutagenesis can be performed to yield point mutations, deletions, or insertions to alter the specific regions of the genes described above. Point mutations can be introduced by a variety of methods including chemical mutagenesis, mutagenic copying methods and site specific mutagenesis methods using
35 synthetic oligonucleotides.

 Cassette mutagenesis methods are conveniently used to introduce point mutations into the specified regions of the PA or LF genes. A double-stranded oligonucleotide region

containing alterations in the specified sequences of the gene is prepared. This oligonucleotide cassette region can be prepared by synthesizing an oligonucleotide with the sequence alteration in residues of the PA or LF gene, annealing to a primer, elongating with the large fragment of DNA polymerase and trimming with BstBI. This double-stranded oligonucleotide is ligated into the BamHI/BstBI fragment from pYS5 and the PpuMI-BamHI fragment from pYS6 to produce an intact recombinant DNA. Other methods of producing the double stranded oligonucleotides and other recombinant DNA vectors can be practiced.

Chemical mutagenesis can be performed using the M13 vector system. A single strand M13 recombinant DNA is prepared containing recombinant PA or LF DNA. Another M13 recombinant containing the same recombinant DNA but in double stranded form is used to prepare a deletion in the targeted region of the gene. This double stranded M13 recombinant is cleaved into a linear molecule with an endonuclease, denatured, and annealed with the single strand M13 recombinant, resulting in a single strand gap in the target region of the PA or LF DNA.

This gapped DNA M13 recombinant is then treated with a compound such as sodium bisulfite to deaminate the cytosine residues in the single strand DNA region to uracil. This results in limited and specific mutations in the single strand DNA region. Finally, the gap in the DNA is filled in by incubation with DNA polymerase, resulting in a U-A base pair to replace a G-C base pair in the in unmutated portion of the gene. Upon replication the new recombinant gene contains T-A base pairs, which are point mutations from the original sequence. Other forms of chemical mutagenesis are also available.

Mutagenic copying of the PA or LF recombinant DNA can be carried out using several methods. For example, a single-stranded gapped DNA region is created as described above. This region is incubated with DNA polymerase I and one or more mutagenic analogs of normal ribonucleoside triphosphates. Copying of the single stranded region with the DNA polymerase

substitutes the mutagenic analogs as the single strand gap region is filled in. Transfection and replication of the resulting DNA results in production of some mutated recombinant DNAs for PA, LF, or EF which can then be selected by cloning. Other mutagenic copying methods can be used.

Point mutations can be introduced into the specified regions of the PA or LF genes by methods using synthetic oligonucleotides for site-specific mutagenesis. PCR copying of the PA or LF genes is performed using oligonucleotide primers covering the specified target regions, and which contain modifications from the wild type sequence in these regions. The PA gene in a pYS5 vector can be PCR amplified using this method to result in mutations in the 164-167 position. PCR amplification can also be used to introduce mutations in the target region of the LF gene.

Synthetic oligonucleotide methods of introducing point mutations can be preformed using heteroduplex DNA. A M13 recombinant DNA vector containing the PA or LF gene is prepared and a single-stranded M13 recombinant is produced. A single strand oligonucleotide containing an alteration in the specified target sequence for the PA or LF gene is annealed to the single strand M13 recombinant to produce a mismatched sequence. Incubation with DNA polymerase I results in a double-stranded M13 recombinant containing base pair mismatches in the specified region of the gene. This M13 recombinant is replicated in a host such as *B. anthracis* or *E. coli* to produce both wild type and mutant M13 recombinants. The mutated M13 recombinants are cloned and isolated. Other vector systems for mutagenesis involving synthetic nucleotides and heteroduplex formation can be applicable.

Expression of Proteins in Prokaryotic Cells

In addition to the use of cloning methods in bacteria such as *Bacillus anthracis* for amplification of cloned sequences, it may be desirable to express the proteins in other prokaryotes. It is possible to recover a functional protein from *E. coli* transformed with an expression plasmid encoding a PA or LF protein. Conveniently, the mutated PA

proteins of the invention were expressed in *B. anthracis* and the LF-fusion proteins were expressed in *E. coli*.

Methods for the expression of cloned genes in bacteria are well known. See Sambrook. To optimize expression of a cloned gene in a prokaryotic system, expression vectors can be constructed which include a promoter to direct mRNA transcription termination. The inclusion of selection markers in DNA vectors transformed in bacteria are useful. Examples of such markers include the genes specifying resistance to ampicillin, tetracycline, or chloramphenicol.

See Sambrook, previously cited, for details concerning selection markers and promoters for use in bacteria such as *E. coli*. In an embodiment of this invention, pY85 is a vector for the subcloning and amplification of desired gene sequences although other vectors could be used.

Strains of *Bacillus anthracis* producing mutated protein(s)

For PA protein production, *B. anthracis* strains cured of both pX01 and pX02 are preferred because they are avirulent. Examples of such strains are UM23Cl-1 and UM44-1C9, obtained from Curtis Thorne, University of Massachusetts. Similar strains can be made by curing of plasmids, as described by P. Mikesell, et al., "Evidence for plasmid-mediated toxin production in *Bacillus anthracis*," *Infect. Immun.* 39:371-376 (1983).

See generally commonly assigned U.S. Patent Application Serial No. 08/042,745, filed April 5, 1993, incorporated by reference herein.

Treatment Methods

A method for delivering a desired activity to a cell is provided. The steps of the method include administering to the cell (a) a protein comprising the translocation domain and the LF binding domain of the native PA protein and a ligand domain, and (b) a product comprising the PA binding domain of the native LF protein and a non-LF activity inducing moiety, whereby the product administered in step (b) is internalized into the cell and performs the activity within the cell.

The method of delivering an activity to a cell can use a ligand domain that is the receptor binding domain of the native PA protein. Other ligand domains are selected for their specificity for a particular cell type or class of cells. The specificity of the PA fusion protein for the targeted cell can be determined using standard methods and as described in Examples 2 and 3.

The method of delivering an activity to a cell can use an activity inducing moiety that is a polypeptide, for example a growth factor, a toxin, an antisense nucleic acid, or a nucleic acid encoding a desired gene product. The actual activity inducing moiety used will be selected based on its functional characteristics, e.g. its activity.

A method of killing a tumor cell in a subject is also provided. The steps of the method can include administering to the subject a first fusion protein comprising the translocation domain and LF binding domain of the native PA protein and a tumor cell specific ligand domain in an amount sufficient to bind to a tumor cell. A second fusion protein is also administered wherein the protein comprises the PA binding domain of the native LF protein and a cytotoxic domain of a non-LF protein in an amount sufficient to bind to the first protein, whereby the second protein is internalized into the tumor cell and kills the tumor cell.

The cytotoxic domain can be a toxin or it can be another moiety not strictly defined as a toxin, but which has an activity that results in cell death. These cytotoxic moieties can be selected using standard tests of cytotoxicity, such as the cell lysis and protein synthesis inhibition assays described in the examples.

The invention further provides a method of killing HIV-infected cells in a subject. The method comprises the steps of administering to the subject a first fusion protein comprising the translocation domain and LF binding domain of the native PA protein and a ligand domain that specifically binds to an HIV protein expressed on the surface of an HIV-infected cell, in an amount sufficient to bind to an HIV-infected cell. The next step is administering to the subject

a second fusion protein comprising the PA binding domain of the native LF protein and a cytotoxic domain of a non-LF protein, in an amount sufficient to bind to the first protein, whereby the second protein is internalized into the HIV-
5 infected cell and kills the HIV-infected cell, thereby preventing propagation of HIV.

Although certain of the methods of the invention have been described as using LF fusion proteins, it will be understood that other LF compositions having chemically
10 attached activity inducing moieties can be used in the methods.

The fusion proteins and other compositions of the inventions can be administered by various methods, e.g., parenterally, intramuscularly or intrapertioneally.

15 The amount necessary can be deduced from other receptor/ligand or antibody/antigen therapies. The amount can be optimized by routine procedures. The exact amount of such LF and PA compositions required will vary from subject to subject, depending on the species, age, weight and general
20 condition of the subject, the severity of the disease that is being treated, the particular fusion protein of composition used, its mode of administration, and the like. Generally, dosage will approximate that which is typical for the administration of cell surface receptor ligands, and will
25 preferably be in the range of about 2 $\mu\text{g/kg/day}$ to 2 mg/kg/day .

Depending on the intended mode of administration, the compounds of the present invention can be in various pharmaceutical compositions. The compositions will include,
30 as noted above, an effective amount of the selected protein in combination with a pharmaceutically acceptable carrier and, in addition, can include other medicinal agents, pharmaceutical agents, carriers, adjuvants, diluents, etc. By
"pharmaceutically acceptable" is meant a material that is not
35 biologically or otherwise undesirable, i.e., the material can be administered to an individual along with the fusion protein or other composition without causing any undesirable biological effects or interacting in a deleterious manner with

any of the other components of the pharmaceutical composition in which it is contained.

Parenteral administration, if used, is generally characterized by injection. Injectables can be prepared in conventional forms, either as liquid solutions or suspensions, solid forms suitable for solution or suspension in liquid prior to injection, or as emulsions. A more recently revised approach for parenteral administration involves use of a slow release or sustained release system, such that a constant level of dosage is maintained. See, e.g., U.S. Patent No. 3,710,795, which is incorporated by reference herein.

Formulations and Administration

Proteins of the invention such as PAHIV are typically mixed with a physiologically acceptable fluid prior to administration to a mammal such as a human. Examples of physiologically acceptable fluids include saline solutions such as normal saline, Ringer's solution, and generally mixtures of various salts including potassium and phosphate salts with or without sugar additives such as glucose. The proteins are administered parenterally with intravenous administration being the most typical route. Either a bolus of the protein in solution or a slow infusion can be administered intravenously. The choice of a bolus or an infusion depends on the kinetics, including the half-life, of the protein in the patient. An appropriate evaluation of the time for delivery of the protein is well within the skill of the clinician.

Patients selected for treatment with PAHIV are infected with HIV-1 and they may or may not be symptomatic. Optimally, the protein would be administered to an HIV-1 infected person who is not yet symptomatic. The dosage range of a protein of the invention such as PAHIV is typically from about 5 to about 25 micrograms per kilogram of body weight of the patient. Usually, the dose is about 10 micrograms per kilogram of body weight of the patient. The dosage is repeated at regular intervals, such as weekly for about 4 to 6 weeks. At that time the clinician may opt to evaluate the

patient's immune status, including immuno-tolerance to the PAHIV, to decide future treatment.

The foregoing description and the following examples are offered primarily for purposes of illustration. It will be readily apparent to those skilled in the art that the operating conditions, materials, procedural steps and other parameters of the system described herein can be further modified or substituted in various ways without departing from the spirit and scope of the invention. For example, although human use has been discussed, veterinary use of the invention is also feasible. For instance, cats suffer from a so-called feline AIDS or feline immunodeficiency virus (FIV). Protective antigen can be altered to include a protease cleavage site specific for FIV. Thus, the invention is not limited by the description and examples, but rather by the appended claims.

EXAMPLE 1

Fusions of Anthrax Toxin Lethal Factor to the ADP-Ribosylation Domain of Pseudomonas Exotoxin Reagents and General Procedures

Restriction endonucleases and DNA modifying enzymes were purchased from GIBCO/BRL, Boehringer Mannheim, or New England Biolabs. Low melting point agarose (Sea Plaque) was obtained from FMC Corp. (Rockland, ME). Oligonucleotides were synthesized on a PCR Mate (Applied Biosystems) and purified on oligonucleotide purification cartridges (Applied Biosystems). The PCR was performed with a DNA amplification reagent (GeneAmp) from Perkin-Elmer Cetus Instruments and a thermal cycler (Perkin-Elmer Cetus). The amplification involved denaturation at 94°C for 1 min, annealing at 55°C for 2.5 min and extension at 72°C for 3 min, for 30 cycles. A final extension was run at 72°C for 7 min. For amplification of PE fragments, 10% formamide was added in the reaction mixture to decrease the effect of high GC content. DNA sequencing reactions were done using the Sequenase version 1.0 from U. S. Biochemical Corp. and DNA sequencing gels were made from Gel Mix 6 from GIBCO/BRL. [³⁵S]deoxyadenosine 5' - [α-

thio]triphosphate and L-[3,4,5-³H]leucine were purchased from Dupont-New England Nuclear. J774A.1 cells were obtained from American Type Culture Collection. Chinese Hamster Ovary (CHO) cells were obtained from Michael Gottesman (National Cancer Institute, National Institutes of Health) (ATCC CCL 61).

Plasmid Construction

Construction of plasmids containing LF-PE fusions was performed as follows. Varying portions of the PE gene were amplified by PCR, ligated in frame to the 3' end of the LF gene, and inserted into the pVEX115 f+T expression vector (provided by V. K. Chaudhary, National Cancer Institute, National Institutes of Health). To construct fusion proteins, the 3'-end of the native LF gene (including codon 776 of the mature protein, specifying Ser) was ligated with the 5'-ends of sequences specifying varying portions of domains II, Ib, and III of PE. The LF gene was amplified from the plasmid pLF7 (Robertson, D. L. and Leppla, S.H. *Gene* 44:71-78, 1986) by PCR using oligonucleotide primers which added *KpnI* and *MluI* sites at the 5' and the 3' ends of the gene, respectively. Similarly, varying portions of the PE gene (provided by David FitzGerald, National Cancer Institute, National Institutes of Health) were amplified by PCR so as to add *MluI* and *EcoRI* sites at the 5' and 3' ends. The PCR product of the LF gene was digested with *KpnI* and the DNA was precipitated. The LF gene was subsequently treated with *MluI*. Similarly, the PCR products of PE amplification were digested with *MluI* and *EcoRI*. The expression vector pVEX115 f+T was cleaved with *KpnI* and *EcoRI* separately and dephosphorylated. This vector has a T7 promoter, OmpA signal sequence, multiple cloning site, and T7 transcription terminator. All the above DNA fragments were purified from low-melting point agarose, a three-fragment ligation was carried out, and the product transformed into *E. coli* DH5 α (ATCC 53868). The four constructs described in this report have the entire LF gene fused to varying portions of PE. The identity of each construct was confirmed by sequencing the junction point using a Sequenase kit (U.S. Biochemical Corp.). For expression, recombinant plasmids were transformed into *E. coli* strain

SA2821 (provided by Sankar Adhya, National Cancer Institute, National Institutes of Health, which is a derivative of BL21(λ DE3) (Studier, F. W. and Moffatt, B.A. *J. Mol. Biol.* 189:113-150, 1986). This strain has the T7 RNA polymerase gene under control of an inducible lac promoter and also contains the *degP* mutation, which eliminates a major periplasmic protease (Strauch et al. *J. Bacteriol.* 171:2689-2696, 1989).

In the resulting plasmids, the LF-PE fusion genes are under control of the T7 promoter and contain an OmpA signal peptide to obtain secretion of the products to the periplasm so as to facilitate purification. The design of the PCR linkers also led to insertion of two non-native amino acids, Thr-Arg, at the LF-PE junction. The four fusions analyzed in this report contain the entire 776 amino acids of mature LF, the two added residues TR (Thr-Arg), and varying portions of PE. In fusion FP33, the carboxyl-terminal end of PE was changed from the native REDLK (Arg-Glu-Asp-Leu-Lys) to LDER, a sequence that fails to cause retention in the ER (endoplasmic reticulum).

Expression and Purification of Fusion Proteins

Fusion proteins produced from pNA2, pNA4, pNA23 and pNA33 were designated FP2, FP4, FP23 and FP33 respectively. *E. coli* strains carrying the recombinant plasmids were grown in super broth (32 g/L Tryptone, 20 g/L yeast extract, 5 g/L NaCl, pH 7.5) with 100 μ g/ml of ampicillin with shaking at 225 rpm at 37°C in 2-L cultures. When A_{600} reached 0.8-1.0, isopropyl-1-thio- β -D-galactopyranoside was added to a final concentration of 1 mM, and cultures were incubated an additional 2 hr. EDTA and 1,10-o-phenanthroline were added to 5 mM and 0.1 mM respectively, and the bacteria were harvested by centrifugation at 4000 x g for 15 min at 4°C. For extraction of the periplasmic contents, cells were suspended in 75 ml of 20% sucrose containing 30 mM Tris and 1 mM EDTA, incubated at 0° for 10 min, and centrifuged at 8000 x g for 15 min at 4°C. Cells were resuspended gently in 50 ml of cold distilled water, kept on ice for 10 min, and the spheroplasts were pelleted. The supernatant was concentrated with

Centriprep-100 units (Amicon) and loaded on a Sephacryl S-200 column (40 x 2 cm) and 1 ml fractions were collected.

Fractions having full length fusion protein as determined by immunoblots were pooled and concentrated as above. Protein was then purified on an anion exchange column (MonoQ HR5/5, Pharmacia-LKB) using a NaCl gradient. The fusion proteins eluted at 280-300 mM NaCl. The proteins were concentrated again on Centriprep-100 (Amicon Division) and the MonoQ chromatography was repeated. Protein concentrations were determined by the bicinchoninic acid method (BCA Protein Assay Reagent, Pierce), using bovine serum albumin as the standard. Proteins were analyzed by polyacrylamide gel electrophoresis in the presence of sodium dodecyl sulfate (SDS). Gels were either stained with Coomassie Brilliant Blue or the proteins were electroblotted to nitrocellulose paper which was probed with polyclonal rabbit antisera to LF or PE (List Biological Laboratories, Campbell, CA). To determine the percent of full length protein, SDS gels stained with Coomassie Brilliant Blue were scanned with a laser densitometer (Pharmacia-LKB Ultrascan XL).

The proteins migrated during gel electrophoresis with molecular masses of more than 106 kDa, consistent with the expected sizes, and immunoblots confirmed that the products had reactivity with antisera to both LF and PE. The fusion proteins differed in their susceptibility to proteolysis as judged by the appearance of smaller fragments on immunoblots, and this led to varying yields of final product. Thus, from 2-L cultures the yields were FP2, 27 μ g; FP4, 87 μ g; FP23, 18 μ g; and FP33, 143 μ g.

Cell Culture Techniques and Protein Synthesis Inhibition Assay

CHO cells were maintained as monolayers in Eagle's minimum essential medium (EMEM) supplemented with 10% fetal bovine serum, 10 mM 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES) (pH 7.3), 2 mM glutamine, penicillin/streptomycin, and non-essential amino acids (GIBCO/BRL). Cells were plated in 24- or 48-well dishes one day before the experiment. After overnight incubation the medium was replaced with fresh medium containing 1 μ g/ml

of PA unless otherwise indicated. Fusion proteins were added to 0.1-1000 ng/ml. All data points were done in duplicate. Cells were further incubated for 20 hr at 37°C in 5% CO₂ atmosphere. The medium was then aspirated and cells were incubated for 2 hr at 37°C with leucine-free medium containing 1 µCi/ml [³H]leucine. Cells were washed twice with medium, cold 10% trichloroacetic acid was added for 30 min, the cells were washed twice with 5% trichloroacetic acid and dissolved in 0.150 ml 0.1 M NaOH. Samples were counted in Pharmacia-LKB 1410 liquid scintillation counter. In experiments to determine if the toxin is internalized through acidified endosomes, 1 µM monensin (Sigma) was added 90 min prior to toxin and was present during all subsequent steps. To verify that the fusion proteins were internalized through the PA receptor, competition with native LF was carried out. PA (0.1 µg/ml) and LF (0.1-10,000 ng/ml) were added to the CHO cells to block the PA receptor and the fusion proteins were added thereafter at concentrations of 100 ng/ml for FP4 and FP23 and 5 ng/ml for FP33. Protein synthesis inhibition was measured after 20 hr as described above.

Cytotoxic Activity of the Fusion Proteins

All four fusion proteins made and purified were toxic to CHO cells. The concentration causing 50% lysis of cultured cells (EC₅₀) values of the proteins were 350, 8, 10, and 0.2 ng/ml for FP2, FP4, FP23 and FP33 respectively (Table 1). These assays were done with PA present at 1 µg/ml, exceeding the K_m of 0.1 µg/ml (100 pM). The fusion proteins had no toxicity even at 1 µg/ml when PA was omitted, proving that internalization of the fusion proteins was occurring through the action of PA and the PA receptor. Native LF has previously been shown to have no short-term toxic effects on CHO cells when added with PA, and therefore was not included in these assays. The fusion protein having only domain III and an altered carboxyl-terminus (FP33) was most active, whereas the one having the intact domains II and III and the native REDLK terminus (FP2) was least active. The other two fusion proteins (FP4 and FP23) had intermediate potencies.

Among proteins having ADP-ribosylation activity, potencies equalling or exceeding 1 pM have previously been found only for native diphtheria and *Pseudomonas* toxins acting on selected cells (Middlebrook, J. L. and Dorlan, R.B. *Can. J. Microbiol.* 23:183-189, 1977) and for fusion proteins of PE and diphtheria toxin when tested on cells containing > 100,000 receptors for the ligand-recognition domain of the fusion (EGF, transferrin, etc.) (Pastan, I. and FitzGerald, D. *Science* 254:1173-1177, 1991; Middlebrook, et al. 1977). For CHO cells, the potency of FP33 ($EC_{50} = 2$ pM) is higher than that of PE itself ($EC_{50} = 420$ pM), even though CHO cells probably have similar numbers of receptors for both PA and PE (approx. 5,000-20,000). If the intracellular trafficking of native PE delivers less than 5% of the molecules to the cytosol, then the 200-fold greater potency of FP33 suggests that the PA/LF system has an inherently high efficiency of delivery to the cytosol.

A comparison of the potencies of the four fusion proteins shows that inclusion of domain II decreases potency. Thus, the fusion with the lowest potency, FP2, was the one containing intact domains II, Ib, and III. In designing the fusion proteins, all or part of PE domain II and Ib was included in several of the constructs because it could not be assumed that the translocation functions possessed by PA and LF would be able to correctly traffic PE domain III to the cytosol. The combination of domains II, Ib, and III, termed PE40, has been used in a large number of toxic hybrid proteins, by fusion to growth factors, monoclonal antibodies, and other proteins (Pastan et al. 1991; Oeltmann, T. N. and Frankel, A. E. *Faseb J.* 5:2334-2337, 1991), and some of these fusions have shown substantial potency. Domain II was found to be essential in these hybrid proteins to provide a translocation function not present in the receptor-binding domain to which it was fused. The potency of many of these PE40 fusion proteins appears to require that they be trafficked through the Golgi and ER and proteolytically activated in the same manner as native PE, so as to achieve delivery of domain III to the cytosol. The fact that

inclusion of the entire domain II in the LF fusion protein FP2 instead decreased activity suggests that internalization of the LF fusions occurs through a different route, one that does not easily accommodate all the sequences in domain II.

5 Evidence that structures within PE residues 251-278 inhibit translocation of the LF fusions comes from the 35-fold lower potency of FP2 compared to FP23. One structure that might inhibit translocation of the fusions is the disulfide loop formed by Cys265 and Cys287. In native PE, this
10 disulfide loop appears to be required for maximum activity. Thus, native PE and TGF- α -PE40 fusions become 10- to 100-fold less toxic if one or both these cysteines are changed to serine. The disulfide loop probably acts to constrain the polypeptide so that Arg276 and Arg279 are susceptible to the
15 intracellular protease involved in the cleavage that precedes translocation. In contrast, the disulfide loop decreases the potency of the LF fusions, perhaps by preventing the unfolding needed for passage through a protein channel, thereby acting in this situation as a "stop transfer" sequence. FP23, which
20 lacks Cys265, would not contain the domain II disulfide, and therefore would not be subject to this effect. LF, like PA and EF, contains no cysteines, and would not be prevented by disulfide loops from the complete unfolding needed to pass through a protein channel. The suggestion that disulfide
25 loops act as stop-transfer signals would predict that the disulfide Cys372-Cys379 in PE domain Ib, which is retained in all four LF fusions would also decrease potency. It should be noted that neither the fusions made here nor the PE40 fusions have been analyzed chemically to determine if the disulfides
30 in domains II and III are actually formed. If the disulfides do form correctly, it would be predicted that the potencies of all of the fusion proteins, and especially that of FP2, would be increased by treatment with reducing agents. These analyses have not yet been performed. This analysis also
35 suggests that future LF fusions might be made more potent by omission of domain Ib.

The other structural feature of PE known to affect intracellular trafficking is the carboxyl terminal sequence,

REDLK, that specifies retention in the ER (Chaudhary et al. 1990; Muro et al. 1987). To determine if the trafficking of the LF fusion proteins was similar to that of PE, two of the fusion proteins were designed so as to differ only in the terminal sequence. Replacement of the native sequence by LDER, one that does not function as an ER retention signal, produced the most toxic of the four fusion proteins, FP33. FP4, identical except that it retained a functional REDLK sequence, was 30-fold less potent. These data suggest that sequestration of the REDLK-ended fusions decreased their access to cytosolic EF-2. The implication is that PE may require the REDLK terminus to be delivered to the ER for an obligatory processing step, but then be limited in its final toxic potential by sequestration from its cytosolic target. Finally, this comparison strongly argues that internalization of the LF fusions does not follow the same path as PE.

In designing the fusion proteins described here it was hoped that they would have cytotoxic activity against cells that are unaffected by anthrax lethal toxin, and this was successfully realized as shown by the data obtained with CHO cells. However, prior knowledge about LF did not provide a basis for predicting whether the constructs would retain toxicity toward mouse macrophages, the only cells known to be rapidly killed by anthrax lethal toxin. Macrophages are lysed by lethal toxin in 90-120 minutes, long before any inhibition of protein synthesis resulting from ADP-ribosylation of EF-2 leads to decreases in membrane integrity or viability. This kinetic difference made it possible to test directly for LF action. As discussed above, the fusion proteins purified to remove the \approx 89-kDa LF species formed by proteolysis were not toxic to J774A.1 macrophages. This shows that attachment of a bulky group to the carboxyl terminus of LF eliminates its normal toxic activity. In the absence of any assay for the putative catalytic activity of LF, it is not possible to determine the cause of the loss of LF activity. The inability of the fusions to lyse J774A.1 cells also argues against proteolytic degradation of the fusions either in the medium during incubation with cells or after internalization.

An important result of the invention described here is the demonstration that the anthrax toxin proteins constitute an efficient mechanism for protein internalization into animal cells. The high potency of the present fusion proteins argues that this system is inherently efficient, as well as being amenable to improvement. The high efficiency results in part from the apparent direct translocation from the endosome, without a requirement for trafficking through other intracellular compartments. In addition to its efficiency, the system appears able to tolerate heterologous polypeptides.

Macrophage Lysis Assay of Fusion Proteins

Fusion proteins were assayed for LF functional activity on J774A.1 macrophage cell line in the presence of 1 μ g/ml PA. One day prior to use, cells were scraped from flasks and plated in 48-well tissue culture dishes. For cytotoxicity tests, the medium was aspirated and replaced with fresh medium containing 1 μ g/ml PA and the LF fusion proteins, and the cells were incubated for 3 hr. All data points were performed in duplicate. To measure the viability of the treated cells, 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide (MTT) was added to the cells to a final concentration of 0.5 mg/ml, and incubation was continued for an additional 45 min to allow the uptake and oxidation of MTT by viable cells. Medium was aspirated and replaced by 200 μ l of 0.5% SDS, 40 mM HCl, 90% isopropanol and the plates were vortexed to dissolve the blue pigment. The MTT absorption was read at 570 nm using a UVmax Kinetic Microplate Reader (Molecular Devices Corp.).

The crude periplasmic extracts from which the fusion proteins were purified caused lysis of J774A.1 macrophages when added with PA, indicating the presence of active LF species, probably formed by proteolysis of the fusion proteins. Purification removed this activity, so that none of the final fusion proteins had this activity. This result showed both that the purified proteins were devoid of full size LF or active LF fragments, and that the lytic activity of LF for macrophages is blocked when residues from PE are fused at its carboxyl terminus.

ADP-Ribosylation Assays

For assaying ADP-ribosylation activity, the method of Collier and Kandel (Collier, R. J. and Kandel, J. J. *Biol. Chem.* 246:1496-1503, 1971) was used with some modification. A wheat germ extract enriched for EF-2 was used in the reaction. Briefly, in a 200- μ L reaction assay, 20 μ L of buffer (500 mM Tris, 10 mM EDTA, 50 mM dithiothreitol and 10 mg/ml bovine serum albumin) was mixed with 30 μ L of EF-2, 130 μ L of H₂O or sample, and 20 μ L of [adenylate-³²P]NAD (0.4 μ Ci per assay, ICN Biochemicals) containing 5 μ M of non-radioactive NAD. Samples were incubated for 20 min at 23°C, the reactions were stopped by adding 1 ml 10% trichloroacetic acid, and the precipitates were collected and washed on GA-6 filters (Gelman Sciences). The filters were washed twice with 70% ethanol, air dried, and the radioactivity measured.

Table 1 shows that all the fusion proteins were equally capable of ADP-ribosylation of EF-2. FP2, which had little cytotoxic activity on CHO cells, still retained full ADP-ribosylation activity. It was also found that treatment with urea and dithiothreitol under conditions that activate the enzymatic activity of native PE, caused no increase in the ADP-ribosylation activity of the fusion proteins, suggesting that the proteins were not folded so as to sterically block the catalytic site.

Effect of Mutant PA on LF-PE Activity

To verify that uptake of the fusion proteins requires PA, the activity of the fusion proteins was measured in the presence of a mutant PA which is apparently defective in internalization. This mutant, PA-S395C, has a serine to cysteine substitution at residue 395 of the mature protein, and retains the ability to bind to receptor, become proteolytically nicked, and bind LF, but is unable to lyse macrophages. When PA-S395C was substituted for native PA in combination with FP33, no inhibition of protein synthesis inhibition was observed. Similar results were obtained when the other three fusion proteins were tested in combination with PA-S395C.

Effect of Monensin on Activity of the Fusion Proteins

To verify that internalization of the fusion proteins was occurring by passage through acidified endosomes in the same manner as native LF, the ability of monensin to protect
5 cells was examined. Addition of monensin to 1 μ M decreased the potency of FP33 by >100-fold. Protection against the other three fusion proteins exceeded 20-fold.

LF Block of LF-PE Fusion Activity

To further verify that the fusion proteins were
10 internalized through the PA receptor, CHO cells were incubated with PA and different amounts of LF to block the receptor and the fusion proteins were added thereafter. Protein synthesis inhibition assays showed that native LF could competitively block LF-PE fusion proteins in a concentration-dependent
15 manner.

The present data suggest that the receptor-bound 63-kDa proteolytic fragment of PA forms a membrane channel and that regions at or near the amino-termini of LF and EF enter this channel first and thereby cross the endosomal membrane,
20 followed by unfolding and transit of the entire polypeptide to the cytosol. This model differs from that for diphtheria toxin in that the orientation of polypeptide transfer is reversed. Since both EF and LF have large catalytic domains, extending to near their carboxyl termini, it appears probable
25 that the entire polypeptide crosses the membrane. In the LF fusion proteins, the attached PE sequences would be carried along with the LF polypeptide in transiting the channel to the cytosol. Thus, the PA63 protein channel must tolerate diverse amino acid residues and sequences. The data presented is
30 consistent with the mechanism of direct translocation of the LF proteins to the cytosol as suggested herein.

TABLE 1 Cytotoxic and catalytic activity of LF-PE fusion proteins

Prot -ein	Amino acid content			Toxicity (EC ₅₀) ^b		ADP- Ribosylation activity (relative)
	LF	Link er	PE	(pM)	ng/ ml	
PE	none	none	1-613	420	23	100 ^c
FP2	776	TR	251-613	2700	350	82
FP4	776	TR	362-613	65	8	105
FP23	776	TR	279-613	70	10	108
FP33	776	TR	362-612 ^a	2	0.2	118

^aREDLK at carboxyl terminus is changed to LDER.

^bData is from this example, except for native PE, which is from data not shown, and is equal to a value previously reported (Moehring, T. J. and Moehring, J. M. Cell 11:447-454, 1977).

^cADP-ribosylation was measured using 30 ng of fusion protein in a final volume of 0.200 ml with 5 μ M NAD. Results were corrected for the molecular weights of the proteins and normalized to PE.

EXAMPLE 2: Residues 1-254 of Anthrax Toxin Lethal Factor are Sufficient to Cause Cellular Uptake of Fused Polypeptides
Reagents and General Procedures

Restriction endonucleases and DNA modifying enzymes
5 were purchased from GIBCO/BRL, Boehringer Mannheim or New
England Biolabs. Low melting point agarose (Sea Plaque) was
obtained from FMC Corporation. Oligonucleotides were
synthesized on a PCR Mate (Applied Biosystems) and purified
10 with Oligonucleotide Purification Cartridges (Applied
Biosystems). Polymerase chain reactions (PCR) were performed
on a thermal cycler (Perkin-Elmer-Cetus) using reagents from
U. S. Biochemical Corp. or Perkin-Elmer-Cetus. DNA was
amplified as described in Example 1. The DNA was sequenced to
15 confirmed the accuracy of all of the constructs described in
the report. SEQUENASE version 2.0 from U. S. Biochemical
Corp. was utilized for the sequencing reactions, and DNA
sequencing gels were made with Gel Mix 8 from GIBCO/BRL.
[³⁵S]dATPαS and L-[3,4,5-³H]leucine were purchased from
20 Dupont-New England Nuclear. Chinese hamster ovary cells (CHO)
were obtained from Michael Gottesman (NCI, NIH). J774A.1
macrophage cells were obtained from American Type Culture
Collection.

Plasmid Construction

Three types of LF protein constructs were made and
25 analyzed in this report. All the constructs were made by PCR
amplification of the desired sequences, using the native LF
gene as template. LF proteins deleted at the amino- or
carboxyl-terminus were constructed by a single PCR
amplification reaction that added restriction sites at the
30 ends for incorporation of the construct into the expression
vector. LF proteins deleted for one or more of the 19-amino
acid repeats that comprise residues 308-383 were constructed
by ligating the products of two separate PCR reactions that
amplified the regions bracketing the deletion. The third
35 group of constructs were fusions of varying portions of the
amino terminus of LF with PE domains Ib and III. Like the
internally-deleted LF proteins, these LF-PE fusions were also
made by ligation of two separate PCR products. In the latter

two types of constructs, the ligation of the PCR products resulted in addition of a linker, ACGCGT, at the junction points. This introduced two non-native residues, Thr-Arg, between the fused domains. The PCR manipulations also added
5 three non-native amino acids, Met-Val-Pro, as an extension to the native amino terminus on all the constructs described in this report. Addition of this sequence is not likely to alter the activity of the constructs (discussed below). It should be noted that the LF-PE fusions described herein contain this
10 three-residue extension.

For PCR reactions to make deletions of 40 and 78 amino acids from the amino-terminus of LF, two different mutagenic oligonucleotide primers were made which were substantially identical to the LF gene template at the intended new termini,
15 and which added *KpnI* sites at their 5'-ends. Another (non-mutagenic) oligonucleotide primer for introduction of a *BamHI* site at the 3' end of LF was prepared. Similarly, to make deletions at the carboxyl-terminus of LF, two different mutagenic primers were used which truncated LF at residues 729
20 and 693 and introduced a *BamHI* site next to the new 3' ends of the LF gene. A second (non-mutagenic) oligonucleotide primer specific for the amino terminus of LF was made which introduced a *KpnI* site at the 5' end of the gene. All of the primers noted above were used in PCR reactions on a pLF7
25 template (Robertson and Leppla, 1986) to synthesize DNA fragments having *KpnI* and *BamHI* sites at their 5' and 3' ends, respectively. The amplified LF DNAs containing the amino- and carboxyl-terminal deletions were digested with the appropriate restriction enzymes. The expression vector pVEX115f+T
30 (provided by V. K. Chaudhary, NCI, NIH) was cleaved sequentially with *KpnI* and *BamHI* and dephosphorylated. This expression vector contains a T7 promoter, an OmpA signal sequence for protein transport to the periplasm, a multiple cloning site that includes *KpnI* and *BamHI* sites, and a T7
35 transcription terminator. The LF and pVEX115f+T DNA fragments were purified from low melting point agarose, ligated overnight, and transformed into *E. coli* DH5 α . Transformants were screened by restriction digestion to identify the desired

recombinant plasmids. Proteins produced by these constructs are designated according to the amino acid residues retained; for example the LF truncated at residue 693 is designated LF¹⁻⁶⁹³. All of the mutant LF proteins described above contain
5 three non-native amino acids, Met-Val-Pro, added to the amino-terminus as a result of the PCR manipulations.

To analyze the role of the repeat region of LF, four different constructs were made: 1., removal of the entire repeat region (LF¹⁻³⁰⁷.TR.LF³⁸⁴⁻⁷⁷⁶), 2., removal of the first
10 repeat (LF¹⁻³⁰⁷.TR.LF³²⁷⁻⁷⁷⁶), 3., removal of the last repeat (LF¹⁻³⁶⁴.TR.LF³⁸⁴⁻⁷⁷⁶), and 4., removal of repeats 2-4 (LF¹⁻³²⁶.TR.LF³⁸⁴⁻⁷⁷⁶). To construct LF¹⁻³⁰⁷.TR.LF³⁸⁴⁻⁷⁷⁶, four different primers were used in two separate PCR reactions. To amplify LF¹⁻³⁰⁷, one oligonucleotide primer was made at the 5'-
15 end of the LF gene which added a *KpnI* site, and a second primer was constructed at the end of residue 307, introducing an *MluI* site. For amplifying LF³⁸⁴⁻⁷⁷⁶, a third primer was made at residue 384 with an added *MluI* site, and the fourth primer was made at the residue 776 which introduced a *BamHI*
20 site at the end. Two PCR amplifications were done using primers one/two and three/four with pLF7 as template (Robertson and Leppla, 1986). The first amplification reaction was digested with *KpnI* and *MluI* separately, and the second amplification reaction was digested with *MluI* and
25 *BamHI*. The expression vector pVEX115f+T was digested separately with *KpnI* and *BamHI* and dephosphorylated. All three fragments were gel purified, ligated overnight at 16°C and transformed into *E. coli* DH5α. The other three constructs were made by similar strategies. Oligonucleotide primers one
30 and four were the same for all four constructs, whereas primers two and three were changed accordingly. All four constructs contain Met-Val-Pro at the amino terminus of LF and Thr-Arg at the site of the repeat region deletion.

To construct LF-PE fusion proteins, fragments of the
35 LF gene extending from the amino terminus to various lengths were amplified from plasmid pLF7 (Robertson and Leppla, 1986) by PCR using a common oligonucleotide primer that added a *KpnI* site at the 5' end and mutagenic primers which added *MluI*

sites at the intended new 3' ends. The PCR products of the LF gene were digested with *KpnI*, the DNAs were precipitated, and subsequently digested with *MluI*. Domains Ib and III of the PE gene (provided by David FitzGerald, NCI, NIH) were amplified by PCR using primers which added *MluI* and *EcoRI* sites at the 5' and 3' ends, respectively. The PCR product of PE was digested with *MluI* and *EcoRI*. Similarly, the expression vector pVEX115f+T was digested with *KpnI* and *EcoRI*. All DNA fragments were purified from low-melting agarose gels, three-fragment ligations were carried out, and the products were transformed into *E. coli* DH5 α . The three constructs described in this example have 254, 198 and 79 amino acids of LF joined with PE domains Ib and III. These fusion proteins are designated LF¹⁻²⁵⁴.TR.PE³⁶²⁻⁶¹³ (SEQ ID NO:10), LF¹⁻¹⁹⁸.TR.PE³⁶²⁻⁶¹³, and LF¹⁻⁷⁹.TR.PE³⁶²⁻⁶¹³, respectively. The proteins retain the native carboxyl-terminal sequence of PE, REDLK. It should be noted that these abbreviations do not specify the entire amino acid content of the proteins, because all the constructs also contain Met-Val-Pro, which was added to the amino-terminus of the LF domain by the PCR manipulations.

Expression and Purification of Deleted LF and Fusion Proteins

Recombinant plasmids were transformed into *E. coli* SA2821 (provided by Sankar Adhya, NCI, NIH), a derivative of BL21(λ DE3) (Studier and Moffatt, 1986) that lacks the proteases encoded by the *lon*, *OmpT*, and *degP* genes, and has the T7 RNA polymerase gene under control of the *lac* promoter (Strauch et al., 1989). Transformants were grown in super broth with 100 μ g/ml ampicillin, with shaking at 225 rpm, 37°C, in 2-L cultures. When A₆₀₀ reached 0.8-1.0, isopropyl-1-thio- β -D-galactopyranoside was added to a final concentration of 1 mM and cultures were incubated for an additional 2 h. EDTA and 1,10-o-phenanthroline were added to 5 and 0.1 mM, respectively, and periplasmic protein was extracted as described in Example 1. The supernatant fluids were concentrated by Centriprep-30 units (Amicon) and proteins were purified to near homogeneity by gel filtration (Sephacryl S-200, Pharmacia-LKB) and anion exchange

chromatography (MonoQ, Pharmacia-LKB) as described in Example 1. To determine the percentage of full length protein, SDS gels stained with Coomassie Brilliant Blue were scanned with a laser densitometer (Pharmacia-LKB Ultrascan XL). Western blots were performed as described previously (Singh et al., 1991).

The LF proteins having terminal deletions and the LF-PE fusion proteins were obtained from periplasmic extracts and purified to near homogeneity by gel filtration and anion exchange chromatography. The migration of the proteins was consistent with their expected molecular weights. Immunoblots confirmed that the LF proteins had reactivity with LF antisera, and the LF-PE fusion proteins had reactivity with both LF and PE antisera. Fusion proteins and terminally-deleted LF proteins differed in their susceptibility to proteolysis as judged by the appearance of peptide fragments on the immunoblots, and this was also reflected in the different amounts of purified proteins obtained. Thus, from 2-L cultures the yields of purified proteins were LF⁴¹⁻⁷⁷⁶, 39 µg; LF⁷⁹⁻⁷⁷⁶, 32 µg; LF¹⁻⁷²⁹, 50 µg; LF¹⁻⁶⁹³, 46 µg; LF¹⁻²⁵⁴.TR.PE³⁶²⁻⁶¹³, 184 µg; LF¹⁻¹⁹⁸.TR.PE³⁶²⁻⁶¹³, 80 µg; LF¹⁻⁷⁹.TR.PE³⁶²⁻⁶¹³, 127 µg.

LF proteins deleted in the repeat region were found to be unstable and full size product could not be purified. Therefore, the activities of these proteins were determined by assay of crude periplasmic extracts, and immunoblots were used to estimate the amount of the full size proteins present.

Cytotoxicity on Macrophages of LF Proteins Having Terminal and Internal Deletions

Deleted LF proteins were assayed for LF functional activity on the J774A.1 macrophage cell line in the presence of native PA as described in Example 1. Briefly, cells were plated in 24- or 48-well dishes in Dulbecco's modified Eagle medium (DMEM) containing 10% fetal bovine serum, and allowed to grow for 18 h. PA (1 µg/ml) and the mutant LF proteins were added and cells were incubated for 3 h. To measure the viability of the treated cells, 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide (MTT) was added to the cells

to a final concentration of 0.5 mg/ml. After incubating for 45 min, the medium was aspirated and cells were dissolved in 90% isopropanol, 0.5% SDS, 40 mM HCl, and read at 540 nm using a UVmax Kinetic Microplate Reader (Molecular Devices Corp.).

5 To determine the extent of essential sequences at the amino terminus of LF, the toxicities of the two LF proteins deleted at the amino-terminus were measured in combination with PA in the macrophage lysis assay. Purified LF⁴¹⁻⁷⁷⁶ and LF⁷⁹⁻⁷⁷⁶ were unable to lyse J774A.1 macrophage cells. This
10 indicates that some portion of the sequence preceding residue 41 is needed to maintain an active LF protein.

To examine the role of the carboxyl terminus of LF, two proteins truncated in this region were prepared and analyzed. The proteins LF¹⁻⁶⁹³ and LF¹⁻⁷²⁹ were assayed on
15 J774A.1 cells and found to be inactive. This is presumed to be due to inactivation of the putative catalytic domain.

To begin study of the role of the repeat region of LF, four constructs were made having deletions in this region. The proteins expressed from these mutants were unstable. Of
20 the four deleted proteins, only LF¹⁻³⁰⁷.TR.LF³²⁷⁻⁷⁷⁶ had immunoreactive material at the position expected of intact fusion protein. The amount of intact LF¹⁻³⁰⁷.TR.LF³²⁷⁻⁷⁷⁶ was similar to that of native LF expressed in the same vector. When these unpurified periplasmic extracts were tested in
25 J774A.1 macrophages, only the native LF control was toxic. LF¹⁻³⁰⁷.TR.LF³²⁷⁻⁷⁷⁶ did not lyse macrophages even when present at 50-fold higher concentration than that of crude periplasmic protein of LF. Conclusions cannot be drawn about the toxicities of the other three constructs because full size
30 fusion proteins were not present in the periplasmic extracts.
Cell Culture Techniques and Protein Synthesis Inhibition Assay of Fusion Proteins

CHO cells were maintained as monolayers in α -modified minimum essential medium (α -MEM) supplemented with 5% fetal
35 bovine serum, 10 mM HEPES (pH 7.3), and penicillin/streptomycin. Protein synthesis assays were carried out in 24- or 48-well dishes as described in Example 1. CHO cells were incubated with PA (0.1 ug/ml) and varying

concentrations of LF, which is expected to block the receptor. Fusion proteins were added at fixed concentrations, as follows: FP4, 100 ng/ml, FP23, 100 ng/ml, and FP33, 5 ng/ml. Cells were incubated for 20 hr and protein synthesis inhibition was evaluated by [³H]leucine incorporation.

Cytotoxicity of the LF-PE Fusion Proteins on CHO Cells

The use of fusion proteins provides a more defined method for measuring the translocation of LF, as demonstrated in Example 1 showing that fusions of LF with domains Ib and III of PE are highly toxic. Translocation of these fusions is conveniently measured because domain III blocks protein synthesis by ADP-ribosylation of elongation factor 2. The new fusions containing varying portions of LF fused to PE domains Ib and III were designed to identify the minimum LF sequence able to promote translocation. The EC₅₀ of LF¹⁻²⁵⁴.TR.PE³⁶²⁻⁶¹³ (SEQ ID NO: 10) was 1.7 ng/ml, whereas LF¹⁻¹⁹⁸.TR.PE³⁶²⁻⁶¹³ and LF¹⁻⁷⁹.TR.PE³⁶²⁻⁶¹³ did not kill 50% of the cells even at a 1200-fold higher concentration. Other constructs were also made and analyzed, containing larger portions of LF fused to PE domains Ib and III, and found those to be equal in potency to LF¹⁻²⁵⁴.TR.PE³⁶²⁻⁶¹³. These results show that residues 1-254 contain all the sequences essential for binding to PA63. The fusion proteins had no toxicity in the absence of PA, proving that their internalization absolutely requires interaction with PA.

Binding of Fusion Proteins and Deleted LF Proteins to PA

Binding of LF proteins to cell bound PA was determined by competition with radiolabeled ¹²⁵I-LF. Native LF was radiolabeled (3.1 x 10⁶ cpm/μg protein) using the Bolton-Hunter reagent. Binding studies employed the L6 rat myoblast cell line, which has approximately twice as many receptors as the J774A.1 macrophage line (Singh et al. 1989). For convenience, cells were chemically fixed by a gentle procedure that preserves the binding activity of the receptor as well as the ability of the cell-surface protease to cleave PA to produce receptor-bound PA63. Assays were carried out in 24-well dishes using cells plated in DMEM with 10% fetal bovine serum one day before the experiment. Cell monolayers

were washed twice with Hanks' balanced salt solution (HBSS) containing 25 mM HEPES and were chemically fixed for 30 min at 23° in 10 mM N-hydroxysuccinimide and 30 mM 1-ethyl-3-[3-dimethyl[aminopropyl] carbodiimide, in buffer containing
5 10 mM HEPES, 140 mM NaCl, 1 mM CaCl₂, and 1 mM MgCl₂. Monolayers were washed with HBSS containing 25 mM HEPES and the fixative was inactivated by incubating 30 min at 23° in DMEM (without serum) containing 25 mM HEPES. Native PA was added at 1 µg/ml in minimum essential medium containing Hanks' salts, 25 mM HEPES, 1% bovine serum albumin, and a total of
10 4.5 mM NaHCO₃. Cells were incubated overnight at room temperature to allow binding and cleavage of PA. Cells were washed twice in HBSS and mutant LF proteins (0-5000 ng/ml) along with 50 ng/ml ¹²⁵I-LF was added to each well. Cells
15 were further incubated for 5 h, washed three times in HBSS, dissolved in 0.5 ml 1 N NaOH, and counted in a gamma counter (Beckman Gamma 9000).

Using this assay, the LF mutant proteins having amino-terminal deletions were found incapable of binding to PA, thereby explaining their lack of toxicity. Carboxyl-terminal
20 deleted LF proteins did bind to PA in a dose dependent manner, although they had slightly lower affinity than LF. The proteins deleted in the repeat region could not be tested for competitive binding because their instability prevented
25 purification of intact protein.

The EC₅₀ for LF¹⁻²⁵⁴.TR.PE³⁶²⁻⁶¹³ binding was found to be 220 ng/ml, which is similar to that of LF, 300 ng/ml. Therefore the binding data correlate well with the toxicity of this construct. In contrast, neither LF¹⁻¹⁹⁸.TR.PE³⁶²⁻⁶¹³ nor
30 LF¹⁻⁷⁹.TR.PE³⁶²⁻⁶¹³ bound to PA63 on cells, thereby explaining their lack of toxicity.

EXAMPLE 3: Construction of Genes Encoding PA Fusion Proteins

The genes encoding PA (or PA truncated at the carboxyl
35 terminus to abrogate binding to the PA receptor) and an alternative targeting moiety (a single-chain antibody, growth factor, or other cell type-specific domain) are spliced using conventional molecular biological techniques. The PA gene is

readily available, and the genes encoding alternative targeting domains are derived as described below.

Single-chain antibodies (sFv)

See Example 4, below.

5 Growth factors and other targeting proteins

The nucleotide sequences of genes encoding a number of growth factors and other proteins that are targeted to specific cell types or classes are reported in freely accessible databases (e.g., GenBank), and in many cases the genes are available. In circumstances where this is not the case, genes can be cloned from genomic or cDNA libraries, using probes based on the known nucleotide sequence of the gene that codes for the growth factor, or derived from a partial amino acid sequence of the protein (see, e.g. Sambrook, supra.). Alternatively, genes encoding the growth factor or other targeting moiety can be produced *de novo* from chemically synthesized overlapping oligonucleotides, using the preferred codon usage of the expression host. For example, the gene for human epidermal growth factor urogastrone was synthesized from the known amino acid sequence of human urogastrone using yeast preferred codons. The cloned DNA, under control of the yeast GAPDH promoter and yeast ADH-1 terminator, expresses a product having the same properties as natural human urogastrone. The product of this synthesized gene is nearly identical to that of the natural urogastrone, the only difference being that the product of the synthetic gene has a tryptophan at amino acid 13, while the other has a tyrosine (Urdea et al. *Proc. Natl. Acad. Sci. USA* 80:7461-7465, 1983).

30 Expression of PA Fusion proteins

Once constructed, genes encoding PA-fusion proteins are expressed in *Bacillus anthracis* and recombinant proteins are purified by one of the following methods: (i) size-based chromatographic separation; (ii) affinity chromatography. In the case of PA-sFv fusions, immobilized metal chelate affinity chromatography may be the purification method of choice, because addition of a string of six histidine residues at the carboxyl terminus of the sFv will have no detrimental effect

on binding to antigen. Additional methods of expression of PA-fusion proteins utilize an *in vitro* rabbit reticulocyte lysate-based coupled transcription/translation system, which has been demonstrated to accurately refold chimeric proteins consisting of an sFv fused to diphtheria toxin, or *Pseudomonas* exotoxin A as demonstrated in Example 4.

Functional testing of PA Fusion proteins

After expression and purification, functionality of PA-fusion proteins are tested by determining their ability to act in concert with an LF-PE fusion protein to inhibit protein synthesis in an appropriate cell line. Using a PA-anti human transferrin receptor sFv fusion as a model, the following properties are examined: (i) Cell type-specificity (protein synthesis should be inhibited in cell lines which express the human transferrin receptor, but not in those which do not); (ii) Independence of toxicity from PA receptor binding (excess free PA should have no effect on toxicity of the PA-sFv/LF-PE complex); (iii) Competitive inhibition by excess free antibody (toxicity should be abrogated in the presence of excess sFv, or the monoclonal antibody from which it was derived). For example such tests are described in Examples 4 and 5. These studies and other studies are used to confirm that PA has been successfully re-routed to an alternative receptor to permit the use of the present anthrax toxin-based cell type-specific cytotoxic agents for the treatment of disease.

EXAMPLE 4: Generating Fusion Proteins with Single-chain Antibodies Reagents

Methionine-free rabbit reticulocyte lysate-based coupled transcription/translation reagents, recombinant ribonuclease inhibitor (rRNasin), and cartridges for the purification of plasmid DNA were purchased from Promega (Madison, WI). Tissue culture supplies were from GIBCO (Grand Island, NY) and Biofluids (Rockville, MD). OKT9 monoclonal antibody was purchased from Ortho Diagnostic Systems (Raritan, NJ). PCR reagents were obtained from by Perkin-Elmer Cetus Instruments (Norwalk, CT), and restriction and nucleic acid modifying enzymes (including M-MLV reverse transcriptase) were

from GIBCO-BRL (Gaithersburg, MD). A GeneClean kit for the recovery of DNA from agarose gels was supplied by BIO 101 (La Jolla, CA). Hybridoma mRNA was isolated using a Fast Trak mRNA isolation kit (Invitrogen, San Diego, CA). All isotopes were purchased from Du Pont-New England Nuclear (Boston, MA), except [Adenylate-³²P]NAD, which was supplied by ICN Biomedicals (Costa Mesa, CA). *Pseudomonas* exotoxin A was obtained from List Biologicals (Campbell, CA). Oligonucleotides were synthesized on a dual column Milligen-Biosearch Cyclone Plus DNA synthesizer (Burlington, MA), and purified using OPC cartridges (Applied Biosystems, Foster City, CA). DNA templates were sequenced using a Sequenase II kit (United States Biochemical Corp., Cleveland, OH), and SDS-polyacrylamide gel electrophoresis (PAGE) was performed using 10-20% gradient gels (Daiichi, Tokyo, Japan). After electrophoresis, gels were fixed in 10% methanol/7% acetic acid, and soaked in autoradiography enhancer (Amplify, Amersham Arlington Heights, IL). After drying, autoradiography was performed overnight using X-OMAT AR2 film (Eastman Kodak, Rochester, NY).

Plasmids

The vector pET-11d is available from Novagen, Inc., Madison, WI. Plasmids were maintained and propagated in *E. coli* strain XL1-Blue (Stratagene, La Jolla, CA).

Cell Lines

K562, a human erythroleukemia-derived cell line [ATCC CCL 243] known to express high levels of the human transferrin receptor at the cell surface, was cultured in RPMI 1640 medium containing 24 mM NaHCO₃, 10% fetal calf serum, 2 mM glutamine, 1 mM sodium pyruvate, 0.1 mM nonessential amino acids, and 10 µg/ml gentamycin. An African green monkey kidney line, Vero (ATCC CCL 31), was grown in Dulbecco's modified Eagle's medium (DMEM) supplemented as indicated above. The OKT9 hybridoma (ATCC CRL 8021), which produces a MoAb (IgG₁) reactive to the human transferrin receptor, was maintained in Iscove's modified Dulbecco's medium containing 20% fetal calf serum, in addition to the supplements described above. All cell lines were cultured at 37°C in a 5% CO₂ humidified atmosphere.

Construction of sFv from Hybridomas

Antibody V_L and V_H genes were cloned using a modification of a previously described technique (Larrick et al. *Biotechniques* 7:360, 1989; Orlandi et al. *Proc. Natl. Acad. Sci. USA* 86:3833, 1989; Chaudhary et al., 1990). Briefly, mRNA was isolated from 1×10^8 antibody producing hybridoma cells, and approximately 3 μ g was reverse transcribed with M-MLV reverse transcriptase, using random hexanucleotides as primers. The resulting cDNA was screened with two sets of PCR primer pairs designed to ascertain from which Kabat gene family the heavy and light chains were derived (Kabat et al. *Sequences of proteins of immunological interest*. Fifth Edition. (Bethesda, Maryland: U.S. Public Health Service, 1991). Having identified the most effective primer pairs, cDNA's encoding V_L and V_H were spliced, separated by a region encoding a 15 amino acid peptide linker, using a previously described PCR technique known as gene splicing by overlap extension (SOE) (Johnson & Bird *Methods Enzymol.* 203:88, 1991). The sFv gene was then cloned into pET-11d, in frame and on the 5'-side of the PE40 gene, such that expression of the construct should generate an sFv-PE40 fusion protein approximately 70 kDa in size.

Design of primers for PCR amplification of V region genes

The first and third complementarity determining regions (CDRs) of terminally rearranged immunoglobulin variable region genes are flanked by conserved sequences (the first framework region, FR1 on the 5' side of CDR1, and the fourth framework region, FR4, on the 3' side of CDR3).

Although murine variable region genes have been successfully cloned, regardless of family, with just two pairs of highly degenerate primers (one pair for V_L and another for V_H) (Gussow et al. *Cold Spring Harbor Symp. Quant. Biol.* 54:265, 1989; Orlandi et al., 1989; Chaudhary et al., 1990; Batra et al., 1991), the method may not be effective in cases where the number of mismatches between primers and the target sequence is extensive. With this in mind, using the Kabat database of murine V gene sequences the present invention provides a set of ten FR1-derived primers (six for V_L and four

for V_H), such that any of the database sequences selected at random would have a maximum of three mismatches with the most homologous primer. This set of primers can be used effectively to clone V region genes from a number of MoAb secreting cell lines.

Assembly of the OKT9 sFv gene

mRNA isolated from the hybridoma secreting the OKT9 MoAb was converted to cDNA as described previously (Larrick et al., 1989; Orlandi et al., 1989; Chaudhary et al., 1990).

Despite the fact that CL-UNI is the partnering oligonucleotide in each case, a product the required size (approximately 400 bp) is not produced by V_L primers IV/VI, IIa or IIb. This suggests that mismatches between these primers and the target sequence were too extensive to allow efficient amplification.

A similar argument can be used to explain the failure of V_H primers I and III to produce the required product. It is clear that primers V_L -I/III and V_H -V are most effective at amplifying the OKT9 V_L and V_H genes respectively. PCR amplified OKT9 V_L and V_H genes were spliced together using the SOE technique, as previously described (Johnson & Bird, 1991). A synthetic DNA sequence encoding a 15 amino acid linker, was inserted between the variable regions; this linker has been used very effectively in the production of functional sFv (Huston et al., 1991; Johnson & Bird, 1991), and appears to allow the variable chains to assume the optimum orientation for antigen binding. Following splicing of V region genes by the SOE procedure, the DNA fragment encoding the OKT9 sFv was electrophoresed through a 1.5% agarose gel, purified by the GeneClean technique, digested with the appropriate pair of restriction enzymes, and cloned into the pET-11d expression vector in frame and on the 5' side of the PE40 gene.

In vitro expression of sFv-PE40 fusion proteins

Plasmid templates were transcribed and translated using a rabbit reticulocyte lysate-based transcription/translation system, according to the instructions of the manufacturer, in 96-well microtiter plate format L-
[35 S]methionine-labeled proteins (for analysis by SDS-PAGE) and unlabeled proteins (for enzymatic analysis and bioassay),

were produced in similar conditions, except that the isotope was replaced with 20 μ M unlabeled L-methionine in the latter case. Control lysate was produced by adding all reagents except plasmid DNA. After translation, unlabeled samples were
5 dialysed overnight at 4°C against phosphate-buffered saline (PBS), pH 7.4 in Spectra/Por 6 MWCO (molecular weight cutoff) 50,000 tubing (Spectrum, Houston, TX).

Constructs incorporating the aberrant kappa transcript will contain a translation termination codon in the V_L chain
10 as previously described, and would therefore be expected to generate a translation product approximately 12 kDa in size. On the other hand, constructs which have incorporated the productive V_L gene contain no such termination codon, and a full-length fusion protein (approximately 70 kDa in size)
15 should be produced.

In vitro expression studies were used to determine the size of the protein encoded by the OKT9 sFv-PE40 gene. The constructs tested in this experiment clearly produce a protein of approximately 70 kDa, indicating that the clones do not
20 contain the aberrant V_L gene, and are devoid of frameshift mutations. Of several OKT9 sFv constructs tested, none apparently incorporated the incorrect VL gene. However, in the case of another sFv generated by this method (1B7 sFv, derived from a MoAb which binds to pertussis toxin), the
25 majority of the clones tested produced a 12 kDa protein, and were found to contain the aberrant transcript on DNA sequencing. It should be noted that the 12kDa fragment is frequently obscured in 10-20% gradient gels by unincorporated ³⁵S-methionine which co-migrates with the dye front.

30 Determination of Protein Concentration

The enzymatic activities of fusion proteins were compared with those of known concentrations of PE in an ADP-ribosyl transferase assay, allowing molarities to be determined (Johnson et al. *J. Biol. Chem.* 263:1295-1399,
35 1988). Samples were adjusted to contain equivalent concentrations of lysate, thus maintaining an identical amount of substrate (elongation factor 2) in all cases.

Protein Synthesis Inhibition Assay for Functional sFv-PE40 Binding

Binding of the OKT9 sFv to the human transferrin receptor was qualitatively determined by assessing the ability of the OKT9 sFv-PE40 fusion protein to inhibit protein synthesis in the K562 cell line. *Pseudomonas* exotoxin A is a bacterial protein which is capable of inhibiting *de novo* protein synthesis in a variety of eukaryotic cell types. The toxin binds to the cell surface, and ultimately translocates to the cytosol where it enzymatically inactivates elongation factor 2. PE40 is a mutant form of exotoxin A which lacks a binding domain, but is enzymatically active, and capable of translocation. Fusion proteins containing PE40 and an alternative binding domain (for example, an sFv to a cell surface receptor) will inhibit protein synthesis in an appropriate cell line only if the sFv binds to a cell-surface antigen which subsequently internalizes into an acidified endosome (Chaudhary et al., 1989). The TfnR is such an antigen, so a qualitative assessment of binding may be determined by measuring the ability of the OKT9 sFv-PE40 fusion protein to inhibit protein synthesis in a cell line like K562, which expresses the TfnR. Protein synthesis inhibition assays were performed as described previously (Johnson et al., 1988). Briefly, samples were serially diluted in ice cold PBS, 0.2% BSA, and 11 μ l volumes were added to the appropriate well of a 96-well microtiter plate (containing 10⁴ cells/100 μ l/well in leucine-free RPMI 1640). After carefully mixing the contents of each well, the plate was incubated for the indicated time at 37°C in a 5% CO₂ humidified atmosphere. Each well was then pulsed with 20 μ l of L-[¹⁴C(U)]leucine (0.1 μ Ci/20 μ l), incubated for 1 hour, and harvested onto glass fiber filters using a PHD cell harvester (Cambridge Technology, Cambridge, MA). Results are expressed as a percentage of the isotope incorporation in cells treated with appropriate concentrations of control dialyzed lysate.

The results of this assay, clearly indicate that OKT9 sFv-PE40 is capable of inhibiting protein synthesis with an IC₅₀ (the concentration of a reagent which inhibits protein

synthesis by 50%) of approximately 2×10^{-9} M. The toxicity of the fusion protein, but not of PE, was abrogated in the presence of excess OKT9 MoAb (12 μ g/ml), indicating that binding is specific for the TfnR. No toxicity was observed when K562 was substituted with Vero (an African Green monkey cell line which expresses the simian version of the transferrin receptor), indicating that the OKT9 sFv retains the human receptor-specific antigen binding properties of the parent antibody.

Having demonstrated binding of the OKT9 sFv to TfnR, its nucleotide sequence was determined using dideoxynucleotide chain-terminating methods, confirming extensive homology with the respective regions of immunoglobulins of known sequence.

EXAMPLE 5: Characterization of single-chain antibody (sFv)-toxin fusion proteins produced in vitro in rabbit reticulocyte lysate

The present invention provides *in vitro* production of proteins containing a toxin domain (derived from Diphtheria toxin (DT) or PE) fused to a domain encoding a single-chain antibody directed against the human transferrin receptor (TfnR). The expression of this antigen on the cell surface is coordinately regulated with cell growth; TfnR exhibits a limited pattern of expression in normal tissue, but is widely distributed on carcinomas and sarcomas (Gatter, et al. *J. Clin. Pathol.* 36:539-545, 1983), and may therefore be a suitable target for immunotoxin-based therapeutic strategies (Johnson, V. G. and Youle, R. J. "Intracellular Trafficking of Proteins" *Cambridge Univ. Press, Cambridge England*, Steer and Hover eds., pp. 183-225; Batra et al., 1991; Johnson et al. 1988).

Proteins consisting of a fusion between an sFv directed against the TfnR and either the carboxyl-terminus 40 kDa of PE, or the DT mutant CRM 107 [S(525)F] were expressed in rabbit reticulocyte lysates, and found to be specifically cytotoxic to K562, a cell line known to express TfnR. In comparison, a chimeric protein consisting of a fusion between a second DT mutant, DTM1 [S(508)F, S(525)F] and the E6 sFv

exhibited significantly lower cytotoxicity. Legal restrictions imposed on manipulating toxin genes *in vivo* previously prevented expression of potentially interesting toxin-containing fusion proteins (*Federal Register* 51(88)(III):16961 and Appendix F:16971); the present invention provides a novel procedure for *in vitro* gene construction and expression which satisfies the regulatory requirements, facilitating the first study of the potential of non-truncated DT mutants in fusion protein ITs. The present data also demonstrates that functional recombinant antibodies can be generated *in vitro*.

Reagents

DT and PE were purchased from List Biologicals (Campbell, CA). Nuclease treated, methionine-free rabbit reticulocyte lysate and recombinant ribonuclease inhibitor (rRNasin) were obtained from Promega (Madison, WI). Tissue culture supplies were from GIBCO (Grand Island, NY) and Biofluids (Rockville, MD). Reagents for PCR were provided by Perkin-Elmer Cetus (Norwalk, CT). Restriction and nucleic acid modifying enzymes were from Stratagene (La Jolla, CA), as was the mCAP kit used to produce capped mRNA *in vitro*. GeneClean and RNaid kits (for the purification of DNA and RNA respectively) were supplied by BIO 101 (La Jolla, CA). L-[³⁵S]methionine, L-[¹⁴C(U)]leucine and 5'-(alpha-thio)-[³⁵S]dATP were from New England Nuclear (Boston, MA). [Adenylate-³²P]NAD was supplied by ICN Biomedicals (Costa Mesa, CA).

Oligonucleotide Synthesis

Oligonucleotides were synthesized (0.2 μM scale), using cyanoethylphosphoramidites supplied by Milligen-Bioscience (Burlington, MA) on a dual column Cyclone Plus DNA synthesizer. Post-synthesis purification was achieved using OPC cartridges (Applied Biosystems, Foster City, CA).

Plasmids

pET-11d was the generous gift of Dr. F. William Studier, Brookhaven National Laboratory (Upton, NY). pHB21-PE40, a derivative of pET-11d containing the gene for PE40, was kindly supplied by Dr. David FitzGerald (NIH, Bethesda,

MD). All plasmids were maintained and propagated in *E. coli* strain XL1-Blue (Stratagene, La Jolla, CA).

Cell Lines

Corynebacterium diphtheriae strain C7₈(β)^{tox+} (ATCC 27012) was obtained from the ATCC (Rockville, MD), and the strain producing the binding-deficient DT mutant CRM 103 was the generous gift of Dr. Neil Groman, University of Washington (Seattle, WA). Both strains were propagated in LB broth.

K562 (a human erythroleukemia-derived cell line, ATCC CCL 243) was cultured in RPMI 1640 medium containing 24 mM NaHCO₃, 10% fetal calf serum, 2 mM glutamine, 1 mM sodium pyruvate, 0.1 mM nonessential amino acids, and 10 μ g/ml gentamycin. Vero (an African green monkey kidney line, ATCC CCL 81) was grown in Dulbecco's modified Eagle's medium supplemented as described above. All eukaryotic cells were cultured at 37°C in a 5% CO₂ humidified atmosphere.

Splicing Genes using PCR

Genes encoding antibody V_L and V_H were spliced, separated by a region encoding a 15 amino acid peptide linker, using a previously described PCR technique known as gene splicing by overlap extension (SOE) (Horton et al. *Gene* 77:61-68, 1989; Horton et al. *Biotechniques* 8:528-535, 1990). For studies requiring *in vitro* expression of PCR products, *tox* gene-derived fragments were linked to those encoding sFv using a similar method, without the use of restriction enzymes.

Construction of Plasmids Encoding Toxin-sFv Fusion Proteins

The gene encoding PE40 was obtained as an insert in pET-11d, and the sFv gene was cloned on the 5' side of this insert as indicated. To clone the gene encoding the DT binding-site mutant DTM1 [S(508)F, S(525)F], genomic DNA was isolated from the *C. diphtheriae* strain which produces CRM 103. DNA was extracted by a modification of the cetyltrimethylammonium bromide extraction procedure (Wilson, K. "Current Protocols in Molecular Biology" Asubel et al. eds. John Wiley & Sons New York, 2.4.1 - 2.4.5, 1988) and subjected to 20 cycles of PCR amplification. Primers were designed to: (i) amplify the 1605 bp region encoding CRM 103, concomitantly mutating the codon at position 525 from TCT to TTT, and (ii)

incorporate restriction sites appropriate for cloning. The mutations present in CRM 107 and CRM 103 were thus combined on a single gene.

In Vitro Transcription of DNA Templates

5 For transcription, DNA templates required a T7 RNA polymerase promoter immediately upstream of the gene of interest (Oakley, J. L. and Coleman, J. E. *Proc. Acad. Sci. U.S.A.* 74:4266-4270, 1977). Such a promoter was conveniently present in pET-11d (Studier et al. *Enzymol* 185:60-89, 1990).
10 In the case of PCR products, the upstream primer (a 57-mer, T7-DT) was used to introduce all of the elements necessary for *in vitro* transcription/translation. T7-DT includes a consensus T7 RNA polymerase promoter, together with the first seven codons of mature DT (Greenfield et al. *Proc. Natl. Acad. Sci. U.S.A.* 80:6853-6857, 1983) immediately preceded by an ATG translation initiation codon in the optimum Kozak context (Kozak, M. J. *Biol. Chem.* 266:19867-19870, 1991).
15 m⁷G(5')ppp(5')G-capped RNA was produced by transcription from linearized plasmids or PCR products using an mCAP kit, according to the manufacturer's protocol. Prior to translation, RNA was purified using an RNaid kit, recovered in nuclease free water, and analyzed by formaldehyde gel electrophoresis.

In Vitro Expression of Fusion Proteins

25 L-[³⁵S]methionine-labelled proteins (for analysis by SDS-PAGE) were produced from capped RNA in methionine-free, nuclease treated rabbit reticulocyte lysate, according to the supplier's instructions. Unlabeled proteins (for bioassay), were produced in similar conditions, except that the isotope
30 was replaced with 20 μ M unlabeled L-methionine. Control lysate was produced by adding all reagents except exogenous RNA. After translation, samples were dialysed overnight at 4°C against PBS, pH 7.4 in Spectra/Por 6 MWCO 50,000 tubing (Spectrum, Houston, TX).

35 Prior to transcription, plasmids were linearized at the BglIII site and treated with proteinase K to destroy ribonucleases that may contaminate the sample. After phenol/chloroform extraction and ethanol precipitation, DNA

was dissolved in nuclease free water to a concentration of approximately 0.2 $\mu\text{g}/\mu\text{l}$. $\text{m}^7\text{G}(5')\text{ppp}(5')\text{G}$ -capped RNA was synthesized by T7 RNA polymerase using the conditions recommended by the manufacturer, and its integrity was confirmed by formaldehyde gel electrophoresis. Capped RNA was translated in a commercially available rabbit reticulocyte lysate, according to the instructions of the manufacturer. It is clear from the gel that the major band in each case has a molecular weight corresponding to that of the protein of interest, and that relatively large molecules (approximately 120 kDa in the case of DTM1-E6 sFv-PE40) can be synthesized in the lysate using the conditions described.

Immediately following translation, samples were extensively dialyzed overnight at 4°C against PBS, pH 7.4. The dialysis step was found to be essential, because non-dialyzed rabbit reticulocyte lysate resulted in the incorporation of significantly lower amounts of ^{14}C -leucine upon assay by protein synthesis inhibition in all cell lines tested. After determining the concentration of the newly synthesized protein using a standard assay for measuring ADP-ribosyltransferase activity (Johnson et al., 1988), the cytotoxic activity of samples was immediately determined.

ADP-ribosyl Transferase Assay

The enzymatic activity (and therefore molarity) of fusion proteins was determined by comparison with DT or PE standard curves, as described previously (Johnson et al., 1988). Appropriate volumes of control lysate were added to each standard curve sample, in order to control for the presence of significant levels of EF-2 in reticulocyte lysate.

Other Methods

SDS-PAGE was performed as previously described (Laemmli, U. K. Nature 227:680-685, 1970), using 10-20% gradient gels (Daiichi, Tokyo, Japan). Once electrophoresis was complete, gels were fixed for 15 minutes in 10% methanol, 7% acetic acid, and then soaked for 30 minutes in autoradiography enhancer (Amplify, Amersham Arlington Heights, IL). After drying, autoradiography was performed overnight using X-OMAT AR2 film (Eastman Kodak, Rochester, NY), in the

absence of intensifying screens. Dideoxynucleotide chain-termination sequencing of double-stranded DNA templates was performed using a Sequenase II kit (United States Biochemical Corp., Cleveland, OH), according to the manufacturer's protocol.

Cytotoxicity of Toxin-sFv Fusion Proteins Expressed in Reticulocyte Lysates

The cytotoxic activity of fusion proteins was determined by their ability to inhibit protein synthesis in relevant cell lines (e.g., K562). Assays were performed as described previously (Johnson et al., 1988). Briefly, samples were serially diluted in ice cold PBS, 0.2% BSA, and 11 μ l volumes were added to the appropriate well of a 96-well microtiter plate (containing 10⁴ cells/well in leucine-free RPMI 1640). After carefully mixing the contents of each well, the plate was incubated for the indicated time at 37°C in a 5% CO₂ humidified atmosphere. Each well was then pulsed with 20 μ l of L-[¹⁴C(U)]leucine (0.1 μ Ci/20 μ l), incubated for 1 hour, and harvested onto glass fiber filters using a PHD cell harvester (Cambridge Technology, Cambridge, MA). Results were expressed as a percentage of the isotope incorporation in cells treated with appropriate concentrations of control dialyzed lysate.

The results of the protein synthesis inhibition assay clearly indicate that PE40-containing fusion proteins synthesized in cell-free reticulocyte lysates are highly cytotoxic to this cell line (IC₅₀ 1 x 10⁻¹⁰ M). In contrast, DTM1-E6 sFv was at least ten-fold less toxic to K562 than the PE40-containing fusion protein, despite the fact that it exhibited ADP-ribosyl transferase activity indistinguishable from that of wt DT synthesized from an equivalent amount of RNA in an identical reticulocyte lysate mix. Since the decreased toxicity of DTM1-E6 sFv is clearly not due to a deficit in enzymatic activity, the binding and/or translocation process is implicated. Possible mechanisms by which the sFv-antigen interaction could be inhibited include: (i) misfolding of the sFv domain or (ii) steric interactions with other regions of the fusion protein preventing close

association of sFv with the TfnR. It is of interest that a tripartite protein, DTM1-E6 sFv-PE40 was significantly cytotoxic to K562 (IC_{50} around 1×10^{-10} M, similar to that of PE40-E6 sFv), and the toxic effect was clearly mediated via the TfnR, since this activity was blocked by addition of excess E6 Mab. Although it is possible that the inclusion of the PE40 moiety at the carboxyl end of the tripartite molecule results in a significant conformational change in domains more proximal to the amino terminus, it seems unlikely that the sFv binding domain of DTM1-E6 is misfolded, or unavailable to interact with the TfnR. Interactions of DTM1-E6 sFv with the cell surface could be measured in a direct binding assay (Greenfield et al. *Science* 238:536-539, 1987), but these studies were not performed in the course of this investigation. Nevertheless, it appears likely that the lack of toxicity of the DTM1-E6 sFv fusion protein is due to a deficit in its translocation function.

The expression system developed is rapid and easy, and facilitates the manipulation of a number of samples at once. No complicated protein purification or refolding procedures are required, and the method can be used to express proteins which, due to restrictions imposed on the manipulation of toxin-encoding genes, could not be produced by more conventional methods. The technique is ideal for ascertaining the suitability of new sFv for IT development; it is theoretically possible to assemble the sFv-encoding gene (and that encoding the IT itself) by splicing of PCR products derived directly from the hybridoma, without the necessity for cloning. This would facilitate the selection of the most promising candidate molecule, prior to investing considerable effort and expense in large scale protein production and purification. Toxins and toxin-containing fusion proteins are proving to be powerful aids in our understanding of receptor mediated endocytosis and intracellular routing, and are providing valuable insight into normal cell function (reviewed in ref. 2). The method described simplifies the generation of such molecules, and facilitates their production and use in

laboratories in which the application of more conventional expression methods would be impractical.

Example 6: Cassette Mutagenesis to Produce PAHIV Mutants.

Three pieces of DNA are joined together. Piece A has
5 vector sequences and encodes the "front half" (5' end of the
gene) of PA protein, B is short piece of DNA (referred to as a
cassette) and encodes a small middle piece of PA protein and
piece C which encodes the "back half" (3' end of the gene) of
PA.

10 PA with alternate HIV-1 cleavage sites were created by
a cassette mutagenesis procedure. Eight deoxyoligonucleotides
were synthesized for construction of cassettes coding for
specifically designed amino acid sequences. All four
cassettes were generated by annealing two synthetic
15 oligonucleotides (primers).

Primer 1A CG CAA GTA TCA CAA AAT TAT CCG ATC GTG CAA AAC ATA CTG CAG G

Q V S O N Y P I V O N I L Q

Primer 1B G TTC CTG CAG TAT GTT TTG CAC GAT CGG ATA ATT TTG TGA TAC TTG

20

Primer 2A CG AAC ACT GCC ACT ATC ATG ATG CAA CGT GGT AAT TTT CTG CAG G

N T A T I M M O R G N F L Q

Primer 2B G TCC CTG CAG AAA ATT ACC ACG TTG CAT CAT GAT AGT GGC AGT GTT

25

Primer 3A CG ACT GTC TCT TTT AAC TTC CCG CAA ATC ACG CTT TGG CTG CAG G

T V S F N F P O I T L W L Q

Primer 3B G TCC CTG CAG CCA AAG CGT GAT TTG CGG GAA GTT AAA AGA GAC AGT

30

Primer 4A CG GGC GGT TCT GCC TTT AAC TTC CCG ATC GTC ATG GGA GGT CTG CAG G

G G S A F N F P I V M G G L Q

Primer 4B G TCC CTG CAG ACC TCC CAT GAC GAT CGG GAA GTT AAA GGC AGA ACC GGT

35

The underlined portion of each protein sequence is
recognized and cleaved by the HIV-1 protease.

Primer pair 1 encodes a protein sequence which
duplicates part of the cleavage site found between the
40 membrane associated protein and the capsid protein.

Primer pair 2 encodes a protein sequence which duplicates part of the cleavage site between the capsid and the nucleocapsid protein.

5 Primer pair 3 encodes a protein sequence which duplicates part of the cleavage site between the protease and the p6 protein. Like the protease, p6 is a portion of the large protein produced by HIV.

10 Primer pair 4 encodes a protein sequence which should be cleaved by the protease. It was created by examining several protein sequences which are recognized by the HIV protease and using the common residues from each sequence. Glycine residues were added to each end to make the molecule more flexible.

15 The mutagenic cassettes were ligated with the BamHI/BstBI fragment from plasmid pYS5 and the PpuMI-BamI-II fragment from plasmid pYS6. Plasmids shown to have correct restriction maps were transformed into the *E. coli dam⁻ dcm⁻* strain GM2163 (available from New England Bio-Labs, Beverly, MA). Unmethylated plasmid DNA was purified from each mutant and used to transform *B. anthracis*. For methods, see Klimpel, 20 et al. *Proc. Natl. Acad. Sci.* 89:10277-10281 (1992). pYS5 and pYS6 construction are described in Singh, et al. *J. Bio. Chem.* 264:19103-19107 (1989).

25 The nucleotide and amino acid sequence of the mature PA protein after alteration with primer set 2 are shown below. Nucleotides residues 482 to 523 were replaced with cassette 2 resulting in replacement of amino acid residues 162-171 of PA with residues NTATIMMQRGNFLO, PAHIV#2. The altered DNA sequence and the new amino acid residues are underlined.

Sequence Range: 1 to 2220

5 GAA GTTAAA CAG GAG AAC CGG TTATTAAAT GAA TCAGAA TCAAGT TCC CAG GGG TTA CTA
CTT CAATTT GTC CTC TTG GCC AATAATTTA CTT AGT CTT AGT TCA AGG GTC CCC AATGAT
Glu Val Lys Gln Glu Asn Arg Leu Leu Asn Glu Ser Glu Ser Ser Ser Gln Gly Leu Leu>

10 GGA TACTAT TTT AGT GAT TTG AATTTTCAA GCA CCCATG GTGGTT ACCTCT TCT ACTACA
CCT ATGATA AAA TCA CTA AAC TTAAAAGTT CGT GGGTAC CAC CAA TGGAGA AGA TGATGT
Gly Tyr Tyr Phe Ser Asp Leu Asn Phe Gln Ala Pro Met Val Val Thr Ser Ser Thr Thr>

15 GGG GATTTA TCT ATT CCT AGT TCTGAGTTA GAA AATATT CCA TCG GAAAAC CAA TATTTT
CCC CTAAAT AGA TAA GGA TCA AGACTCAAT CTT TTATAA GGTAGC CTT TTG GTT ATAAAA
Gly Asp Leu Ser Ile Pro Ser Ser Glu Leu Glu Asn Ile Pro Ser Glu Asn Gln Tyr Phe>

20 CAA TCTGCT ATT TGG TCA GGA TTTATCAAAGTT AAGAAG AGTGAT GAATAT ACA TTTGCT
GTT AGACGA TAA ACC AGT CCT AAATAGTTT CAA TTC TTC TCACTA CTTATA TGT AAACGA
Gln Ser Ala Ile Trp Ser Gly Phe Ile Lys Val Lys Lys Ser Asp Glu Tyr Thr Phe Ala>

25 ACT TCCGCT GAT AAT CAT GTA ACAATGTGGGTA GATGAC CAAGAAGTGATT AAT AAAGCT
TGA AGGCGA CTA TTA GTA CAT TGTTACACC CAT CTACTG GTT CTT CACTAA TTA TTTCGA
Thr Ser Ala Asp Asn His Val Thr Met Trp Val Asp Asp Gln Glu Val Ile Asn Lys Ala>

30 TCT AATTCT AAC AAA ATC AGA TTAGAAAAA GGA AGATTA TAT CAAATAAAAA ATT CAATAT
AGA TTAAGA TTG TTT TAG TCT AATCTTTTT CCT TCTAAT ATAGTT TATTTT TAA GTTATA
Ser Asn Ser Asn Lys Ile Arg Leu Glu Lys Gly Arg Leu Tyr Gln Ile Lys Ile Gln Tyr>

35 CAA CGAGAA AAT CCT ACT GAA AAAGGATTG GAT TTCAAG TTG TACTGGACC GAT TCTCAA
GTT GCTCTT TTA GGA TGA CTT TTTCTAAC CTA AAGTTC AACATGACCTGG CTA AGAGTT
Gln Arg Glu Asn Pro Thr Glu Lys Gly Leu Asp Phe Lys Leu Tyr Trp Thr Asp Ser Gln>

40 AAT AAAAAA GAA GTG ATT TCT AGTGATAAC TTA CAATTG CCAGAA TTAAAA CAA AAATCT
TTA TTTTTT CTT CAC TAA AGA TCACTATTGAAT GTTAAC GGT CTT AATTTT GTT TTTAGA
Asn Lys Lys Glu Val Ile Ser Ser Asp Asn Leu Gln Leu Pro Glu Leu Lys Gln Lys Ser>

45 TCGAAC ACTGCCACTATCATGATG CAA CGTGGT AAT TTTCTG CAG GGA CCTACG GTTCCA
AGCTTG TGACGGTGA TAGTACTAC GTTGCA CCA TTA AAAAGAC GTC CCT GGATGC CAAGGT
Ser Asn Thr Ala Thr Ile Met Met Gln Arg Gly Asn Phe Leu Gln Gly Pro Thr Val Pro>

50 GAC CGTGAC AAT GAT GGA ATC CCTGATTCA TTA GAGGTA GAAGGATATACG GTT GATGTC
CTG GCACTG TTA CTA CCT TAG GGA CTAAGT AAT CTCCAT CTT CCT ATATGC CAA CTACAG
Asp Arg Asp Asn Asp Gly Ile Pro Asp Ser Leu Glu Val Glu Gly Tyr Thr Val Asp Val>

55 AAA AATAAA AGA ACT TTT CTT TCACCATGGATT TCTAAT ATT CAT GAAAAG AAA GGATTA
TTT TTATTT TCT TGA AAA GAA AGTGGTACC TAA AGATTA TAAGTACTTTTC TTT CCTAAT
Lys Asn Lys Arg Thr Phe Leu Ser Pro Trp Ile Ser Asn Ile His Glu Lys Lys Gly Leu>

720

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ACC AAATAT AAA TCA TCT CCT GAAAAATGG AGC ACGGCT TCTGAT CCGTAC AGT GATTTC
TGG TTTATA TTT AGT AGA GGA CTTTTTACCTCG TGCCGA AGACTAGGCATG TCA CTAAAG
5 Thr LysTyr Lys Ser Ser ProGluLysTrpSer ThrAla SerAspProTyr Ser AspPhe>

780

*

GAA AAGGTT ACA GGA CGG ATT GATAAGAATGTA TCACCA GAGGCAAGACAC CCC CTTGTG
10 CTT TTCCAA TGT CCT GCC TAA CTATTCTTACAT AGTGGT CTC CGTTCTGTG GGG GAACAC
Glu LysVal Thr Gly Arg Ile AspLysAsnVal SerProGluAlaArgHis Pro LeuVal>

840

*

GCA GCTTAT CCG ATT GTA CAT GTAGATATGGAG AATATT ATTCTCTCAAAA AAT GAGGAT
15 CGT CGAATA GGC TAA CAT GTA CATCTATAC CTC TTATAA TAAGAGAGTTTT TTA CTCCTA
AlaAlaTyr Pro Ile Val His ValAspMet Glu AsnIle IleLeuSerLys Asn GluAsp>

900

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CAA TCCACA CAG AAT ACT GAT AGTGAAACGAGA ACAATA AGTAAAAATACT TCT ACAAGT
20 GTT AGGTGT GTC TTA TGA CTA TCACTTTGC TCT TGT TAT TCA TTT TTATGA AGA TGT TCA
Gln SerThr GlnAsn Thr Asp SerGluThr Arg Thr Ile SerLysAsnThr Ser ThrSer>

960

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AGG ACACAT ACT AGT GAA GTA CATGGAAATGCA GAAAGTG CATGCGTCGTTT TTT GATATT
25 TCC TGTGTA TGA TCA GTT CAT GTACCTTTACGT CTTTAC GTACGCAGCAAGAAA CTATAA
Arg ThrHis Thr Ser Glu Val HisGlyAsnAla GluVal HisAlaSer Phe Phe AspIle>

1020

*

GGT GGGAGT GTA TCT GCA GGA TTTAGTAATTCG AATTCA AGTACG GTCGCA ATT GATCAT
35 CCA CCCTCA CAT AGA CGT CCT AAATCATTAAAGC TTAAGT TCATGC CAGCGT TAA CTAGTA
Gly GlySer Val Ser Ala Gly PheSerAsnSer AsnSer SerThrValAla Ile AspHis>

1080

*

TCA CTATCT CTA GCA GGG GAA AGAACTTGG GCT GAAACA ATGGGT TTAAAT ACC GCTGAT
40 AGT GATAGA GAT CGT CCC CTT TCTTGAACC CGA CTTTGT TACCCAATTTA TGG CGACTA
Ser LeuSer LeuAla Gly GluArgThrTrpAla GluThr MetGlyLeuAsnThr AlaAsp>

1140

*

ACA GCAAGA TTA AAT GCC AAT ATTAGATATGTA AATACT GGGACGGCTCCAATC TACAAC
45 TGT CGTTCT AAT TTA CGG TTA TAATCTATA CAT TTATGA CCC TGC CGAGGT TAG ATGTTG
Thr AlaArg LeuAsnAla Asn IleArgTyr Val AsnThr GlyThrAlaPro Ile TyrAsn>

1200

*

GTG TTACCA ACG ACT TCG TTA GTGTTAGGAAAA AATCAA ACA CTC GCGACA ATT AAAGCT
50 CAC AATGGT TGC TGA AGC AAT CACAATCCTTTT TTAGTT TGTGAG CGCTGT TAA TTTCGA
Val LeuPro Thr Thr Ser LeuValLeuGlyLys AsnGln ThrLeuAlaThr Ile LysAla>

1260

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AAG GAAAC CAA TTA AGT CAA ATACTTGCA CCT AATAAT TATTATCCTTCT AAA AACTTC
55 TTC CTTTGT GTT AAT TCA GTT TATGAACGTGGA TTATTA ATAATAGGAAGATT TTGAAC
Lys GluAsn Gln Leu Ser Gln IleLeuAla Pro AsnAsnTyrTyr ProSer Lys AsnLeu>

1320

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GCG CCAATC GCA TTA AAT GCA CAAGACGATTTT AGTTCT ACTCCAATTACAATG AATTAC
65 CGC GGTTAG CGT AAT TTA CGT GTTCTGTCTAAAG TCAAGA TGAGGT TAATGT TAC TTAATG
Ala ProIleAla LeuAsnAla GlnAspAspPhe SerSer ThrProIleThr Met AsnTyr>

1440
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GGG AATATA GCA ACA TAC AAT TTTGAAAT GGA AGAGTG AGGGTG GATACA GGC TCGAAC
CCC TTATAT GCT TGT ATG TTA AAAC TTTTA CCT TCTCAC TCC CAC CTATGT CCG AGCTTG
5 Gly Asn Ile Ala Thr Tyr Asn Phe Glu Asn Gly Arg Val Arg Val Asp Thr Gly Ser Asn

1500
*
TGG AGTGAA GTG TTA CCG CAA ATT CAAGAA ACA ACT GCA CGTATCATT TTT AAT GGAAAA
10 ACC TCACTT CAC AAT GGC GTT TAAGTTC TTT TGT TGACGT GCATAG TAAAAA TTA CCTTTT
Trp Ser Glu Val Leu Pro Gln Ile Gln Glu Thr Thr Ala Arg Ile Ile Phe Asn Gly Lys

1560
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GAT TTAAAT CTG GTA GAA AGG CGGATAGCG GCG GTTAAT CCTAGT GATCCATTA GAAACG
15 CTA AATTTA GAC CAT CTT TCC GCCTATCGC CGC CAATTA GGATCA CTAGGT AAT CTTTGC
Asp Leu Asn Leu Val Glu Arg Arg Ile Ala Ala Val Asn Pro Ser Asp Pro Leu Glu Thr

1620
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20 TGA TTTGGC CTA TAC TGT AAT TTTCTTCGG GAA TTTTAT CGTAAACCTAAATTG CTTGGC
Thr Lys Pro Asp Met Thr Leu Lys Glu Ala Leu Lys Ile Ala Phe Gly Phe Asn Glu Pro

1680
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25 TTA CCTTTG AAT GTT ATA GTT CCCTTTCTG TAT TGGCTT AAAC TAAAAATTA AAG CTAGTT
Asn Gly Asn Leu Gln Tyr Gln Gly Lys Asp Ile Thr Glu Phe Asp Phe Asn Phe Asp Gln

1740
*
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30 GTT TGTAGA GTT TTA TAG TTC TTAGTCAAT CGC CTTAAT TTGCGTTGATTG TAT ATATGA
Gln Thr Ser Gln Asn Ile Lys Asn Gln Leu Ala Glu Leu Asn Ala Thr Asn Ile Tyr Thr

1800
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GTA TTAGAT AAAATC AAA TTA AATGCAAAAATG AATATT TTAATAAGAGAT AAA CGTTTT
35 CAT AATCTA TTT TAG TTT AAT TTACGTTTT TAC TTATAA AAT TAT TCT CTA TTT GCAAAA
Val Leu Asp Lys Ile Lys Leu Asn Ala Lys Met Asn Ile Leu Ile Arg Asp Lys Arg Phe

1860
*
CAT TATGAT AGAAAT AAC ATAGCAGTTGGG GCG GATGAG TCAGTAGTTAAG GAG GCTCAT
40 GTA ATACTA TCT TTA TTG TAT CGTCAACCC CGC CTA CTC AGTCAT CAATTC CTC CGAGTA
His Tyr Asp Arg Asn Asn Ile Ala Val Gly Ala Asp Glu Ser Val Val Lys Glu Ala His

1920
*
AGA GAAGTA ATT AAT TCG TCAACAGAGGGA TTA TTGTTA AATATTGATAAG GAT ATAAGA
45 TCT CTT CAT TAATTA AGC AGT TGTCTCCCT AAT AACAAT TTATAACTATTC CTA TATTCT
Arg Glu Val Ile Asn Ser Ser Thr Glu Gly Leu Leu Leu Asn Ile Asp Lys Asp Ile Arg

1980
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AAA ATATTA TCAGGT TAT ATT GTAGAAATT GAA GATACT GAAGGG CTTAAA GAA GTTATA
50 TTT TATAAT AGT CCAATA TAA CATCTTTAA CTT CTATGA CTTCCC GAATTT CTT CAATAT
Lys Ile Leu Ser Gly Tyr Ile Val Glu Ile Glu Asp Thr Glu Gly Leu Lys Glu Val Ile

2040
*
AAT GACAGA TAT GAT ATG TTG AATATTCT AGT TTACGG CAAGATGGAAAA ACA TTTATA
55 TTA CTGTCT ATA CTA TAC AAC TTATAAGATCA AATGCC GTTCTA CCTTTT TGT AAATAT
Asn Asp Arg Tyr Asp Met Leu Asn Ile Ser Ser Leu Arg Gln Asp Gly Lys Thr Phe Ile

2100

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5 GAT TTTAAA AAA TAT AAT GAT AAATTACCG TTA TATATA AGTAAT CCCAAT TAT AAGGTA
CTA AAATTT TTT ATA TTA CTA TTTAATGGCAAT ATATAT TCATTAGGGTTAATA TTCCAT
Asp PheLys Lys Tyr Asn Asp LysLeuProLeuTyrIle SerAsnProAsnTyr LysVal

2160

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10 AAT GTATAT GCT GTT ACT AAA GAAAACACT ATT ATTAAT CCTAGTGAGAAT GGG GATACT
TTA CATATA CGA CAA TGA TTT CTTTGTGATAA TAATTA GGATCACTCTTACCC CTATGA
Asn ValTyr Ala Val Thr Lys GluAsnThrIle IleAsn ProSerGluAsnGly AspThr

2220

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15 AGT ACCAAC GGG ATC AAG AAA ATTTTAATCTTTTCTAAA AAAGGCTATGAGATA GGATAA
TCA TGGTTG CCC TAG TTC TTT TAAAATTAGAAA AGATTT TTTCCGATACTC TAT CCTATT
Ser ThrAsnGly Ile Lys Lys IleLeuIle Phe SerLys LysGlyTyrGluIle Gly***

20 The above procedure was followed for PAHIV#1, 3 and 4.

Example 7: Cleavage of Mutant PAHIV Proteins in vitro.

The mutated proteins were treated with purified HIV-1 protease and evaluated for their degree of cleavage with respect to time. The purified protease was obtained from the NIH AIDS Research and Reference Reagent Program, Division of AIDS, NIAID, Bethesda, MD. Alternatively, the protease can be purified following the method of Louis, et al., *Euro. J. Biochem.*, 199:361 (1991).

30 Extended incubation (12 hours) of PA or the mutated PA proteins with the purified HIV-1 protease resulted in the appearance of two additional protein fragments that were not anticipated. These two fragments are approximately 53 kilodaltons and 30 kilodaltons in size. This may represent cleavage of PA and mutant PA proteins at a site recognized by the HIV-1 protease between PA residues Y²⁵⁹ and P²⁶⁰. The residues around this cleavage site, ²⁵⁶VAAYPIVHV²⁶⁴, have not previously been identified as a potential HIV-1 protease cleavage site.

40 Incubation of RAW 264.7 cells (ATCC No. TIB 71) with lethal factor (LF) and HIV-1 protease-cleaved PAHIV#1 or PAHIV#4 caused cell death, demonstrating that the mutated PA proteins are capable of binding to LF and thus the toxic LF/PE fusion proteins. PAHIV, PAHIV#2 and PAHIV#3 have not yet been tested.

Example 8: Evaluation of cytotoxic agents in cell cultures.

The ability of the PA constructs containing the HIV-1-protease cleavage site to promote killing of HIV-1 infected cells is being evaluated in COS-1 cells (ATCC No. CRL 1650) transfected with the vector HIV-gpt. When COS cells are transfected with this plasmid vector they express all the genes for the production of HIV-1 virus particles except the envelope protein, gp160 (Page, K.A., et al., 1990. *J. Virol.* 64:5270-5276). Without the envelope protein the particles are not infectious. These cells express the HIV-1 proteases and properly cleave the viral protein gp55 to gp24 (Page, K.A., et al., 1990. *J. Virol.* 64:5270-5276). These properties make the transfected cells an excellent model system in which to evaluate the ability of protein constructs of the invention to eliminate HIV-1 infected cells from culture.

The COS-1 cells were transfected with the plasmid vector and the resulting cultures are being selected for stable transfectants. The mutated PA proteins (PAHIV#1, PAHIV#2, PAHIV#3 and PAHIV#4) are added to the culture media of growing HIV-gpt transfected COS-1 cells in the presence of the lethal factor fusion protein FP53 (Arora, N. et al. *J. Biol. Chem.* 267:15542 (1992)). Only cells which properly cleave the mutated PA proteins are able to bind the toxin LF fusion protein. The cultures are evaluated for protein expression (an indirect measure of viability) after 36 hours (Arora, N. and S. H. Leppla. 1992. *J. Biol. Chem.* 268:3334).

Example 9: Treatment of an HIV-1 infected patient.

A human patient who is infected with HIV-1 is selected for treatment. Although infected, this particular patient is asymptomatic. The patient weighs 70 kilograms. A dose of 10 micrograms per kilogram or 700 micrograms of a PAHIV in normal saline is prepared. This dosage is injected into the patient intravenously as a bolus. The dose is repeated weekly for a total of 4 to 6 dosages. The patient is evaluated regularly, such as weekly, in terms of his symptoms, physical exam and laboratory analysis according to the clinician's judgment. Tests of particular interest include the patient's complete

blood count and examination for the presence of HIV infection. The treatment regimen can be repeated with or without alterations at the discretion of the clinician.

Incorporated by reference/paragraph before claims

5 Unless defined otherwise, all technical and scientific terms used herein have the same meaning as commonly understood by one of ordinary skill in the art to which this invention belongs. Although any methods and materials similar or
10 equivalent to those described can be used in the practice or testing of the present invention, the preferred methods and materials are now described. All publications and patent documents referenced in this application are incorporated herein by reference.

15 It is understood that the examples and embodiments described herein are for illustrative purposes only and that various modifications or changes in light thereof will be suggested to persons skilled in the art and are to be included within the spirit and purview of this application and scope of the appended claims.

SEQUENCE LISTING

(1) GENERAL INFORMATION:

(i) APPLICANT: Leppla, Stephen H.
Klimpel, Kurt R.
Arora, Naveen
Singh, Yogendra
Nichols, Peter J.

(ii) TITLE OF INVENTION: ANTHRAX TOXIN FUSION PROTEINS AND
RELATED METHODS

(iii) NUMBER OF SEQUENCES: 31

(iv) CORRESPONDENCE ADDRESS:

(A) ADDRESSEE: TOWNSEND and TOWNSEND KHOURIE and CREW
(B) STREET: Steuart Street Tower, 20th Floor, One Market
Plaza
(C) CITY: San Francisco
(D) STATE: CA
(E) COUNTRY: USA
(F) ZIP: 94105

(v) COMPUTER READABLE FORM:

(A) MEDIUM TYPE: Floppy disk
(B) COMPUTER: IBM PC compatible
(C) OPERATING SYSTEM: PC-DOS/MS-DOS
(D) SOFTWARE: PatentIn Release #1.0, Version #1.25

(vi) CURRENT APPLICATION DATA:

(A) APPLICATION NUMBER: US
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(C) CLASSIFICATION:

(viii) ATTORNEY/AGENT INFORMATION:

(A) NAME: Weber, Kenneth A.
(B) REGISTRATION NUMBER: 31,677
(C) REFERENCE/DOCKET NUMBER: 15280-115

(ix) TELECOMMUNICATION INFORMATION:

(A) TELEPHONE: (415) 543-9600
(B) TELEFAX: (415) 543-5043

(2) INFORMATION FOR SEQ ID NO:1:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 3291 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(iii) HYPOTHETICAL NO

(iv) ANTI-SENSE: NO

(vi) ORIGINAL SOURCE:

(A) ORGANISM: Bacillus anthracis

(ix) FEATURE:

(A) NAME/KEY: CDS
(B) LOCATION: 580..2907
(D) OTHER INFORMATION: /product= "Lethal Factor"

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:1:

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5	GCAAATGATA AATGGGCTTT AAACAAACT AATGAAATAA TCTACAAATG GAATTTCTCC	120
	AGTTTTAGAT TAAACCATAC CAAAAAATC ACACTGTCAA GAAAAATGAT AGAATCCCTA	180
10	CACATAATTAA CATAACCAA TTGGTAGTTA TAGGTAGAAA CTTATTTATT TCTATAATAC	240
	CATGCAAAAA AGTAAATATT CTGTTCCATA CTATTTTAGT AAATTATTTA GCAAGTAAAT	300
	TTTGGTGTAT AAACAAAGTT TATCTTAATA TAAAAAATTA CTTTACTTTT ATACAGATTA	360
15	AAATGAAAAA TTTTATATGA CAAGAAATAT TGCCTTTAAT TTATGAGGAA ATAAGTAAAA	420
	TTTTCTACAT ACTTTATTTT ATTGTTGAAA TGTTCACCTA TAAAAAAGGA GAGATTAAAT	480
20	ATGAATATAA AAAAAGAATT TATAAAAGTA ATTAGTATGT CATGTTTAGT AACAGCAATT	540
	ACTTTGAGTG GTCCCGTCTT TATCCCCCTT GTACAGGGG GCG GGC GGT CAT GGT	594
		Ala Gly Gly His Gly
		1 5
25	GAT GTA GGT ATG CAC GTA AAA GAG AAA GAG AAA AAT AAA GAT GAG AAT	642
	Asp Val Gly Met His Val Lys Glu Lys Glu Lys Asn Lys Asp Glu Asn	
		10 15 20
30	AAG AGA AAA GAT GAA GAA CGA AAT AAA ACA CAG GAA GAG CAT TTA AAG	690
	Lys Arg Lys Asp Glu Glu Arg Asn Lys Thr Gln Glu Glu His Leu Lys	
		25 30 35
35	GAA ATC ATG AAA CAC ATT GTA AAA ATA GAA GTA AAA GGG GAG GAA GCT	738
	Glu Ile Met Lys His Ile Val Lys Ile Glu Val Lys Gly Glu Glu Ala	
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40	GTT AAA AAA GAG GCA GCA GAA AAG CTA CTT GAG AAA GTA CCA TCT GAT	786
	Val Lys Lys Glu Ala Ala Glu Lys Leu Leu Glu Lys Val Pro Ser Asp	
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45	GTT TTA GAG ATG TAT AAA GCA ATT GGA GGA AAG ATA TAT ATT GTG GAT	834
	Val Leu Glu Met Tyr Lys Ala Ile Gly Gly Lys Ile Tyr Ile Val Asp	
		70 75 80 85
50	GGT GAT ATT ACA AAA CAT ATA TCT TTA GAA GCA TTA TCT GAA GAT AAG	882
	Gly Asp Ile Thr Lys His Ile Ser Leu Glu Ala Leu Ser Glu Asp Lys	
		90 95 100
55	AAA AAA ATA AAA GAC ATT TAT GGG AAA GAT GCT TTA TTA CAT GAA CAT	930
	Lys Lys Ile Lys Asp Ile Tyr Gly Lys Asp Ala Leu Leu His Glu His	
		105 110 115
60	TAT GTA TAT GCA AAA GAA GGA TAT GAA CCC GTA CTT GTA ATC CAA TCT	978
	Tyr Val Tyr Ala Lys Glu Gly Tyr Glu Pro Val Leu Val Ile Gln Ser	
		120 125 130
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	Glu Ile Gly Lys Ile Leu Ser Arg Asp Ile Leu Ser Lys Ile Asn Gln	
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	Pro Tyr Gln Lys Phe Leu Asp Val Leu Asn Thr Ile Lys Asn Ala Ser	
		170 175 180
80	GAT TCA GAT GGA CAA GAT CTT TTA TTT ACT AAT CAG CTT AAG GAA CAT	1170

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5	CCC	ACA	GAC	TTT	TCT	GTA	GAA	TTC	TTG	GAA	CAA	AAT	AGC	AAT	GAG	GTA	1218
	Pro	Thr	Asp	Phe	Ser	Val	Glu	Phe	Leu	Glu	Gln	Asn	Ser	Asn	Glu	Val	
			200					205					210				
10	CAA	GAA	GTA	TTT	GCG	AAA	GCT	TTT	GCA	TAT	TAT	ATC	GAG	CCA	CAG	CAT	1266
	Gln	Glu	Val	Phe	Ala	Lys	Ala	Phe	Ala	Tyr	Tyr	Ile	Glu	Pro	Gln	His	
			215				220					225					
15	CGT	GAT	GTT	TTA	CAG	CTT	TAT	GCA	CCG	GAA	GCT	TTT	AAT	TAC	ATG	GAT	1314
	Arg	Asp	Val	Leu	Gln		Tyr	Ala	Pro	Glu	Ala	Phe	Asn	Tyr	Met	Asp	
	230					235					240					245	
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	Lys	Phe	Asn	Glu		Glu	Ile	Asn	Leu	Ser		Glu	Glu	Glu	Leu	Lys	
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25	CAA	CGG	ATG	CTG	TCA	AGA	TAT	GAA	AAA	TGG	GAA	AAG	ATA	AAA	CAG	CAC	1410
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30	TAT	CAA	CAC	TGG	AGC	GAT	TCT	TTA	TCT	GAA	GAA	GGA	AGA	GGA	CTT	TTA	1458
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			280					285					290				
35	AAA	AAG	CTG	CAG	ATT	CCT	ATT	GAG	CCA	AAG	AAA	GAT	GAC	ATA	ATT	CAT	1506
	Lys	Lys	Leu	Gln	Ile	Pro	Ile	Glu	Pro	Lys	Lys	Asp	Asp	Ile	Ile	His	
			295			300						305					
40	TCT	TTA	TCT	CAA	GAA	GAA	AAA	GAG	CTT	CTA	AAA	AGA	ATA	CAA	ATT	GAT	1554
	Ser	Leu	Ser	Gln	Glu	Glu	Lys	Glu	Leu	Leu	Lys	Arg	Ile	Gln	Ile	Asp	
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45	AGT	AGT	GAT	TTT	TTA	TCT	ACT	GAG	GAA	AAA	GAG	TTT	TTA	AAA	AAG	CTA	1602
	Ser	Ser	Asp	Phe	Leu	Ser	Thr	Glu	Glu	Lys	Glu	Phe	Leu	Lys	Lys	Leu	
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50	CAA	ATT	GAT	ATT	CGT	GAT	TCT	TTA	TCT	GAA	GAA	GAA	AAA	GAG	CTT	TTA	1650
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	Asn	Arg	Ile	Gln	Val	Asp	Ser	Ser	Asn	Pro	Leu	Ser	Glu	Lys	Glu	Lys	
			360					365					370				
60	GAG	TTT	TTA	AAA	AAG	CTG	AAA	CTT	GAT	ATT	CAA	CCA	TAT	GAT	ATT	AAT	1746
	Glu	Phe	Leu	Lys	Lys	Leu	Lys	Leu	Asp	Ile	Gln	Pro	Tyr	Asp	Ile	Asn	
			375				380					385					
65	CAA	AGG	TTG	CAA	GAT	ACA	GGA	GGG	TTA	ATT	GAT	AGT	CCG	TCA	ATT	AAT	1794
	Gln	Arg	Leu	Gln	Asp	Thr	Gly	Gly	Leu	Ile	Asp	Ser	Pro	Ser	Ile	Asn	
			390				395				400					405	
70	CTT	GAT	GTA	AGA	AAG	CAG	TAT	AAA	AGG	GAT	ATT	CAA	AAT	ATT	GAT	GCT	1842
	Leu	Asp	Val	Arg	Lys	Gln	Tyr	Lys	Arg	Arg	Ile	Gln	Asn	Ile	Asp	Ala	
					410					415					420		
75	TTA	TTA	CAT	CAA	TCC	ATT	GGA	AGT	ACC	TTG	TAC	AAT	AAA	ATT	TAT	TTG	1890
	Leu	Leu	His	Gln	Ser	Ile	Gly	Ser	Thr	Leu	Tyr	Asn	Lys	Ile	Tyr	Leu	
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	Leu	Val	Asp	Ser	Thr	Asp	Asn	Thr	Lys	Ile	Asn	Arg	Gly	Ile	Phe	Asn	

	455	460	465	
5	GAA TTC AAA AAA AAT TTC AAA TAT AGT ATT TCT AGT AAC TAT ATG ATT Glu Phe Lys Lys Asn Phe Lys Tyr Ser Ile Ser Ser Asn Tyr Met Ile 470 475 480 485			2034
10	GTT GAT ATA AAT GAA AGG CCT GCA TTA GAT AAT GAG CGT TTG AAA TGG Val Asp Ile Asn Glu Arg Pro Ala Leu Asp Asn Glu Arg Leu Lys Trp 490 495 500			2082
15	AGA ATC CAA TTA TCA CCA GAT ACT CGA GCA GGA TAT TTA GAA AAT GGA Arg Ile Gln Leu Ser Pro Asp Thr Arg Ala Gly Tyr Leu Glu Asn Gly 505 510 515			2130
20	AAG CTT ATA TTA CAA AGA AAC ATC GGT CTG GAA ATA AAG GAT GTA CAA Lys Leu Ile Leu Gln Arg Asn Ile Gly Leu Glu Ile Lys Asp Val Gln 520 525 530			2178
25	ATA ATT AAG CAA TCC GAA AAA GAA TAT ATA AGG ATT GAT GCG AAA GTA Ile Ile Lys Gln Ser Glu Lys Glu Tyr Ile Arg Ile Asp Ala Lys Val 535 540 545			2226
30	GTG CCA AAG AGT AAA ATA GAT ACA AAA ATT CAA GAA GCA CAG TTA AAT Val Pro Lys Ser Lys Ile Asp Thr Lys Ile Gln Glu Ala Gln Leu Asn 550 555 560 565			2274
35	ATA AAT CAG GAA TGG AAT AAA GCA TTA GGG TTA CCA AAA TAT ACA AAG Ile Asn Gln Glu Trp Asn Lys Ala Leu Gly Leu Pro Lys Tyr Thr Lys 570 575 580			2322
40	CTT ATT ACA TTC AAC GTG CAT AAT AGA TAT GCA TCC AAT ATT GTA GAA Leu Ile Thr Phe Asn Val His Asn Arg Tyr Ala Ser Asn Ile Val Glu 585 590 595			2370
45	AGT GCT TAT TTA ATA TTG AAT GAA TGG AAA AAT AAT ATT CAA AGT GAT Ser Ala Tyr Leu Ile Leu Asn Glu Trp Lys Asn Asn Ile Gln Ser Asp 600 605 610			2418
50	CTT ATA AAA AAG GTA ACA AAT TAC TTA GTT GAT GGT AAT GGA AGA TTT Leu Ile Lys Lys Val Thr Asn Tyr Leu Val Asp Gly Asn Gly Arg Phe 615 620 625			2466
55	GTT TTT ACC GAT ATT ACT CTC CCT AAT ATA GCT GAA CAA TAT ACA CAT Val Phe Thr Asp Ile Thr Leu Pro Asn Ile Ala Glu Gln Tyr Thr His 630 635 640 645			2514
60	CAA GAT GAG ATA TAT GAG CAA GTT CAT TCA AAA GGG TTA TAT GTT CCA Gln Asp Glu Ile Tyr Glu Gln Val His Ser Lys Gly Leu Tyr Val Pro 650 655 660			2562
65	GAA TCC CGT TCT ATA TTA CTC CAT GGA CCT TCA AAA GGT GTA GAA TTA Glu Ser Arg Ser Ile Leu Leu His Gly Pro Ser Lys Gly Val Glu Leu 665 670 675			2610
70	AGG AAT GAT AGT GAG GGT TTT ATA CAC GAA TTT GGA CAT GCT GTG GAT Arg Asn Asp Ser Glu Gly Phe Ile His Glu Phe Gly His Ala Val Asp 680 685 690			2658
75	GAT TAT GCT GGA TAT CTA TTA GAT AAG AAC CAA TCT GAT TTA GTT ACA Asp Tyr Ala Gly Tyr Leu Leu Asp Lys Asn Gln Ser Asp Leu Val Thr 695 700 705			2706
80	AAT TCT AAA AAA TTC ATT GAT ATT TTT AAG GAA GAA GGG ACT AAT TTA Asn Ser Lys Lys Phe Ile Asp Ile Phe Lys Glu Glu Gly Ser Asn Leu 710 715 720 725			2754
85	ACT TCG TAT GGG AGA ACA AAT GAA GCG GAA TTT TTT GCA GAA GCC TTT Thr Ser Tyr Gly Arg Thr Asn Glu Ala Glu Phe Phe Ala Glu Ala Phe 730 735 740			2802

75

AGG TTA ATG CAT TCT ACG GAC CAT GCT GAA CGT TTA AAA GTT CAA AAA 2850
 Arg Leu Met His Ser Thr Asp His Ala Glu Arg Leu Lys Val Gln Lys
 745 750 755

5 AAT GCT CCG AAA ACT TTC CAA TTT ATT AAC GAT CAG ATT AAG TTC ATT 2898
 Asn Ala Pro Lys Thr Phe Gln Phe Ile Asn Asp Gln Ile Lys Phe Ile
 760 765 770

10 ATT AAC TCA TAAGTAATGT ATTAAAAATT TTCAAATGGA TTTAATAATA 2947
 Ile Asn Ser
 775

15 ATAATAATAA TAATAATAAC GGGACCAGCC ATTATGAAGC AACTAATTCT AGACTTGATA 3007
 GTAATTCTTG GGAAGCACCA GATAGTGTA AAGGTGGCAT TGCCAGAATG ATATTTTATG 3067
 TGTTCTGTAG ATATGAAGGC AAAACAATG ATCCTGACCT AGAACTTAAT GATAATGTTA 3127
 20 TTAATAATTT AATGCCTTTT ATAGGAATAT TAGTAAAAGT GCCGAAAAGA TCCTGTTGCA 3187
 AAGCTTTTAA AGAACATATT ATTCTATCAA GTGGCTGTAT ATTTGTGTGA ATTTTCAATA 3247
 AATTTTGTA TTAAGCATAC GTCAAAAAAC CGAAATCTGA GCTC 3291

25 (2) INFORMATION FOR SEQ ID NO:2:

(i) SEQUENCE CHARACTERISTICS:
 (A) LENGTH: 776 amino acids
 (B) TYPE: amino acid
 (D) TOPOLOGY: linear

30 (ii) MOLECULE TYPE: protein

35 (xi) SEQUENCE DESCRIPTION: SEQ ID NO:2:

Ala Gly Gly His Gly Asp Val Gly Met His Val Lys Glu Lys Glu Lys
 1 5 10 15

40 Asn Lys Asp Glu Asn Lys Arg Lys Asp Glu Glu Arg Asn Lys Thr Gln
 20 25 30

Glu Glu His Leu Lys Glu Ile Met Lys His Ile Val Lys Ile Glu Val
 35 40 45

45 Lys Gly Glu Glu Ala Val Lys Lys Glu Ala Ala Glu Lys Leu Leu Glu
 50 55 60

50 Lys Val Pro Ser Asp Val Leu Glu Met Tyr Lys Ala Ile Gly Gly Lys
 65 70 75 80

Ile Tyr Ile Val Asp Gly Asp Ile Thr Lys His Ile Ser Leu Glu Ala
 85 90 95

55 Leu Ser Glu Asp Lys Lys Lys Ile Lys Asp Ile Tyr Gly Lys Asp Ala
 100 105 110

Leu Leu His Glu His Tyr Val Tyr Ala Lys Glu Gly Tyr Glu Pro Val
 115 120 125

60 Leu Val Ile Gln Ser Ser Glu Asp Tyr Val Glu Asn Thr Glu Lys Ala
 130 135 140

65 Leu Asn Val Tyr Tyr Glu Ile Gly Lys Ile Leu Ser Arg Asp Ile Leu
 145 150 155 160

Ser Lys Ile Asn Gln Pro Tyr Gln Lys Phe Leu Asp Val Leu Asn Thr
 165 170 175

Ile Lys Asn Ala Ser Asp Ser Asp Gly Gln Asp Leu Leu Phe Thr Asn
 180 185 190
 5 Gln Leu Lys Glu His Pro Thr Asp Phe Ser Val Glu Phe Leu Glu Gln
 195 200 205
 Asn Ser Asn Glu Val Gln Glu Val Phe Ala Lys Ala Phe Ala Tyr Tyr
 210 215 220
 10 Ile Glu Pro Gln His Arg Asp Val Leu Gln Leu Tyr Ala Pro Glu Ala
 225 230 235 240
 Phe Asn Tyr Met Asp Lys Phe Asn Glu Gln Glu Ile Asn Leu Ser Leu
 245 250 255
 15 Glu Glu Leu Lys Asp Gln Arg Met Leu Ser Arg Tyr Glu Lys Trp Glu
 260 265 270
 Lys Ile Lys Gln His Tyr Gln His Trp Ser Asp Ser Leu Ser Glu Glu
 275 280 285
 Gly Arg Gly Leu Leu Lys Lys Leu Gln Ile Pro Ile Glu Pro Lys Lys
 290 295 300
 25 Asp Asp Ile Ile His Ser Leu Ser Gln Glu Glu Lys Glu Leu Leu Lys
 305 310 315 320
 Arg Ile Gln Ile Asp Ser Ser Asp Phe Leu Ser Thr Glu Glu Lys Glu
 325 330 335
 30 Phe Leu Lys Lys Leu Gln Ile Asp Ile Arg Asp Ser Leu Ser Glu Glu
 340 345 350
 Glu Lys Glu Leu Leu Asn Arg Ile Gln Val Asp Ser Ser Asn Pro Leu
 355 360 365
 35 Ser Glu Lys Glu Lys Glu Phe Leu Lys Lys Leu Lys Leu Asp Ile Gln
 370 375 380
 40 Pro Tyr Asp Ile Asn Gln Arg Leu Gln Asp Thr Gly Gly Leu Ile Asp
 385 390 395 400
 Ser Pro Ser Ile Asn Leu Asp Val Arg Lys Gln Tyr Lys Arg Asp Ile
 405 410 415
 45 Gln Asn Ile Asp Ala Leu Leu His Gln Ser Ile Gly Ser Thr Leu Tyr
 420 425 430
 Asn Lys Ile Tyr Leu Tyr Glu Asn Met Asn Ile Asn Asn Leu Thr Ala
 435 440 445
 50 Thr Leu Gly Ala Asp Leu Val Asp Ser Thr Asp Asn Thr Lys Ile Asn
 450 455 460
 55 Arg Gly Ile Phe Asn Glu Phe Lys Lys Asn Phe Lys Tyr Ser Ile Ser
 465 470 475 480
 Ser Asn Tyr Met Ile Val Asp Ile Asn Glu Arg Pro Ala Leu Asp Asn
 485 490 495
 60 Glu Arg Leu Lys Trp Arg Ile Gln Leu Ser Pro Asp Thr Arg Ala Gly
 500 505 510
 Tyr Leu Glu Asn Gly Lys Leu Ile Leu Gln Arg Asn Ile Gly Leu Glu
 515 520 525
 65 Ile Lys Asp Val Gln Ile Ile Lys Gln Ser Glu Lys Glu Tyr Ile Arg
 530 535 540

77

Ile Asp Ala Lys Val Val Pro Lys Ser Lys Ile Asp Thr Lys Ile Gln
 545 550 555 560
 5 Glu Ala Gln Leu Asn Ile Asn Gln Glu Trp Asn Lys Ala Leu Gly Leu
 565 570 575
 Pro Lys Tyr Thr Lys Leu Ile Thr Phe Asn Val His Asn Arg Tyr Ala
 580 585 590
 10 Ser Asn Ile Val Glu Ser Ala Tyr Leu Ile Leu Asn Glu Trp Lys Asn
 595 600 605
 Asn Ile Gln Ser Asp Leu Ile Lys Lys Val Thr Asn Tyr Leu Val Asp
 610 615 620
 15 Gly Asn Gly Arg Phe Val Phe Thr Asp Ile Thr Leu Pro Asn Ile Ala
 625 630 635 640
 20 Glu Gln Tyr Thr His Gln Asp Glu Ile Tyr Glu Gln Val His Ser Lys
 645 650 655
 Gly Leu Tyr Val Pro Glu Ser Arg Ser Ile Leu Leu His Gly Pro Ser
 660 665 670
 25 Lys Gly Val Glu Leu Arg Asn Asp Ser Glu Gly Phe Ile His Glu Phe
 675 680 685
 Gly His Ala Val Asp Asp Tyr Ala Gly Tyr Leu Leu Asp Lys Asn Gln
 690 695 700
 30 Ser Asp Leu Val Thr Asn Ser Lys Lys Phe Ile Asp Ile Phe Lys Glu
 705 710 715 720
 35 Glu Gly Ser Asn Leu Thr Ser Tyr Gly Arg Thr Asn Glu Ala Glu Phe
 725 730 735
 Phe Ala Glu Ala Phe Arg Leu Met His Ser Thr Asp His Ala Glu Arg
 740 745 750
 40 Leu Lys Val Gln Lys Asn Ala Pro Lys Thr Phe Gln Phe Ile Asn Asp
 755 760 765
 Gln Ile Lys Phe Ile Ile Asn Ser
 770 775

(2) INFORMATION FOR SEQ ID NO:3:

(i) SEQUENCE CHARACTERISTICS:

- 50 (A) LENGTH: 4235 base pairs
 (B) TYPE: nucleic acid
 (C) STRANDEDNESS: single
 (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

55

(iii) HYPOTHETICAL: NO

(iv) ANTI-SENSE: NO

60

(vi) ORIGINAL SOURCE:

- (A) ORGANISM: *Bacillus anthracis*

(ix) FEATURE:

65

- (A) NAME/KEY: CDS
 (B) LOCATION: 1891..4095
 (D) OTHER INFORMATION: /product= "Protective Antigen"

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:3:

	AAGCTTCTGT CATTCTGTAAT TTCAAAATAG AACGTAAATT TAGACTTCTC ATCATTAAAA	60
	ATGAAAAATC TTATCTTTTT GATTCTATTG TATATTTTAA TTAAGGTGTT TAATAGTTAG	120
5	AAAAGACAGT TGATGCTATT ACTCCAGATA AAATATAGCT AACCATAAAT TTATTAAAGA	180
	AACCTTGTTG TTCTAAATAA TGATTTTGTG GATTCCGGAA TAGATACTGG TGAGTTAGCT	240
10	CTAATTTTAT AGTGATTAA CTAACAATTT ATAAAGCAGC ATAATTCAAA TTTTAAATTT	300
	GATTTTTCCT GAAGCATAGT ATAAAAGAGT CAAGGTCTTC TAGACTTGAC TCTTGGAATC	360
	ATTAGGAATT AACAATATAT ATAATGCGCT AGACAGAATC AAATTAAATG CAAAAATGAA	420
15	TATTTTAGTA AGAGATCCAT ATCATTATGA TAATAACGGT AATATTGTAG GGGTTGATGA	480
	TTCATATTTA AAAAACGCAT ATAAGCAAAT ACTTAATTGG TCAAGCGATG GAGTTTCTTT	540
20	AAATCTAGAT GAAGATGTAA ATCAAGCACT ATCTGGATAT ATGCTTCAA TAAAAAACC	600
	TTCAAACCAC CTAACAAACA GCCCAGTTAC AATTACATTA GCAGGCAAGG ACAGTGGTGT	660
	TGGAGAATTG TATAGAGTAT TATCAGATGG AGCAGGATTC CTGGATTTCATAAAGTTTGA	720
25	TGAAAATTGG CGATCATTAG TAGATCCTGG TGATGATGTT TATGTGTATG CTGTTACTAA	780
	AGAAGATTTT AATGCAGTTA CTCGAGATGA AAATGGTAAT ATAGCGAATA AATTAAAAAA	840
30	CACCTTAGTT TTATCGGGTA AAATAAAAGA AATAAACATA AAAACTACAA ATATTAATAT	900
	ATTTGTAGTT TTTATGTTTA TTATATACCT CCTATTTTAT ATTATTAGTA GCACAGTTTT	960
	TGCAATCAT GTAATTGTAT ACTTATCTAT GTAGAGGTAT CACAACCTAT GAATAGTGTA	1020
35	TTTTATTGAA CGTTGGTTAG CTTGGACAGT TGTATGGATA TGCATACTTT ATAACGTATA	1080
	AAATTTACAG CACCACAATA AACTAATTT AACAAAAACA AAAACACACC TAAGATCATT	1140
40	CAGTTCTTTT AATAAGGAGC TGCCACCAA GCTAAACCTA AATAATCTTT GTTTCACATA	1200
	AGGTTTTTTT CTAAATATAC AGTGTAAGTT ATTGTGAATT TAACCAGTAT ATATTAAAA	1260
	TGTTTTATGT TAACAAATTA AATTGTAAAA CCCCTCTTAA GCATAGTTAA GAGGGGTAGG	1320
45	TTTTAAATTT TTTGTTGAAA TTAGAAAAAA TAATAAAAAA ACAACCTAT TTTCTTTCAG	1380
	GTTGTTTTTG GGTACAAAA CAAAAAGAAA ACATGTTTCA AGGTACAATA ATTATGGTTC	1440
50	TTTAGCTTTC TGTAAACAG CCTTAATAGT TGGATTATG ACTATTAAAG TTAGTATACA	1500
	GCATACACAA TCTATTGAAG GATATTATTA ATGCAATTCC CTAAAAATAG TTTTGTATAA	1560
	CCAGTTCTTT TATCCGAAC TATACACGTA TTTTAGCATA ATTTTAAATG TATCTTCAAA	1620
55	AACAGCTTCT GTGTCCTTTT CTATTAAACA TATAAATCT TTTTATGTT ATATATTTAT	1680
	AAAAGTCTG TTTAAAAAGC CAAAAATAA TAATTATCTC TTTTATTTA TATTATATG	1740
60	AACTAAAGT TTATTAAAT CAATATAATA TAAATTTAAT TTTATACAAA AAGGAGAACG	1800
	TATATGAAAA AACGAAAAGT GTTAATACCA TTAATGGCAT TGTCTACGAT ATTAGTTTCA	1860
	AGCACAGGTA ATTTAGAGGT GATTCAGGCA GAA GTT AAA CAG GAG AAC CGG TTA	1914
65	Glu Val Lys Gln Glu Asn Arg Leu	
	1 5	
	TTA AAT GAA TCA GAA TCA AGT TCC CAG GGG TTA CTA GGA TAC TAT TTT	1962
	Leu Asn Glu Ser Glu Ser Ser Ser Gln Gly Leu Leu Gly Tyr Tyr Phe	
	10 15 20	

	AGT GAT TTG AAT TTT CAA GCA CCC ATG GTG GTT ACC TCT TCT ACT ACA	2010
	Ser Asp Leu Asn Phe Gln Ala Pro Met Val Val Thr Ser Ser Thr Thr	
	25 30 35 40	
5	GGG GAT TTA TCT ATT CCT AGT TCT GAG TTA GAA AAT ATT CCA TCG GAA	2058
	Gly Asp Leu Ser Ile Pro Ser Ser Glu Leu Glu Asn Ile Pro Ser Glu	
	45 50 55	
10	AAC CAA TAT TTT CAA TCT GCT ATT TGG TCA GGA TTT ATC AAA GTT AAG	2106
	Asn Gln Tyr Phe Gln Ser Ala Ile Trp Ser Gly Phe Ile Lys Val Lys	
	60 65 70	
15	AAG AGT GAT GAA TAT ACA TTT GCT ACT TCC GCT GAT AAT CAT GTA ACA	2154
	Lys Ser Asp Glu Tyr Thr Phe Ala Thr Ser Ala Asp Asn His Val Thr	
	75 80 85	
20	ATG TGG GTA GAT GAC CAA GAA GTG ATT AAT AAA GCT TCT AAT TCT AAC	2202
	Met Trp Val Asp Asp Gln Glu Val Ile Asn Lys Ala Ser Asn Ser Asn	
	90 95 100	
25	AAA ATC AGA TTA GAA AAA GGA AGA TTA TAT CAA ATA AAA ATT CAA TAT	2250
	Lys Ile Arg Leu Glu Lys Gly Arg Leu Tyr Gln Ile Lys Ile Gln Tyr	
	105 110 115 120	
30	CAA CGA GAA AAT CCT ACT GAA AAA GGA TTG GAT TTC AAG TTG TAC TGG	2298
	Gln Arg Glu Asn Pro Thr Glu Lys Gly Leu Asp Phe Lys Leu Tyr Trp	
	125 130 135	
35	ACC GAT TCT CAA AAT AAA AAA GAA GTG ATT TCT AGT GAT AAC TTA CAA	2346
	Thr Asp Ser Gln Asn Lys Lys Glu Val Ile Ser Ser Asp Asn Leu Gln	
	140 145 150	
40	TTG CCA GAA TTA AAA CAA AAA TCT TCG AAC TCA AGA AAA AAG CGA AGT	2394
	Leu Pro Glu Leu Lys Gln Lys Ser Ser Asn Ser Arg Lys Lys Arg Ser	
	155 160 165	
45	ACA AGT GCT GGA CCT ACG GTT CCA GAC CGT GAC AAT GAT GGA ATC CCT	2442
	Thr Ser Ala Gly Pro Thr Val Pro Asp Arg Asp Asn Asp Gly Ile Pro	
	170 175 180	
50	GAT TCA TTA GAG GTA GAA GGA TAT ACG GTT GAT GTC AAA AAT AAA AGA	2490
	Asp Ser Leu Glu Val Glu Gly Tyr Thr Val Asp Val Lys Asn Lys Arg	
	185 190 195 200	
55	ACT TTT CTT TCA CCA TGG ATT TCT AAT ATT CAT GAA AAG AAA GGA TTA	2538
	Thr Phe Leu Ser Pro Trp Ile Ser Asn Ile His Glu Lys Lys Gly Leu	
	205 210 215	
60	ACC AAA TAT AAA TCA TCT CCT GAA AAA TGG AGC ACG GCT TCT GAT CCG	2586
	Thr Lys Tyr Lys Ser Ser Pro Glu Lys Trp Ser Thr Ala Ser Asp Pro	
	220 225 230	
65	TAC AGT GAT TTC GAA AAG GTT ACA GGA CGG ATT GAT AAG AAT GTA TCA	2634
	Tyr Ser Asp Phe Glu Lys Val Thr Gly Arg Ile Asp Lys Asn Val Ser	
	235 240 245	
70	CCA GAG GCA AGA CAC CCC CTT GTG GCA GCT TAT CCG AAT GTA CAT GTA	2682
	Pro Glu Ala Arg His Pro Leu Val Ala Ala Tyr Pro Ile Val His Val	
	250 255 260	
75	GAT ATG GAG AAT ATT ATT CTC TCA AAA AAT GAG GAT CAA TCC ACA CAG	2730
	Asp Met Glu Asn Ile Ile Leu Ser Lys Asn Glu Asp Gln Ser Thr Gln	
	265 270 275 280	
80	AAT ACT GAT AGT GAA ACG AGA ACA ATA AGT AAA AAT ACT TCT ACA AGT	2778
	Asn Thr Asp Ser Glu Thr Arg Thr Ile Ser Lys Asn Thr Ser Thr Ser	
	285 290 295	
85	AGG ACA CAT ACT AGT GAA GTA CAT GGA AAT GCA GAA GTG CAT GCG TCG	2826

80

	Arg	Thr	His	Thr	Ser	Glu	Val	His	Gly	Asn	Ala	Glu	Val	His	Ala	Ser	
				300					305					310			
5	TTC	TTT	GAT	ATT	GGT	GGG	AGT	GTA	TCT	GCA	GGA	TTT	AGT	AAT	TCG	AAT	2874
	Phe	Phe	Asp	Ile	Gly	Gly	Ser	Val	Ser	Ala	Gly	Phe	Ser	Asn	Ser	Asn	
			315					320					325				
10	TCA	AGT	ACG	GTC	GCA	ATT	GAT	CAT	TCA	CTA	TCT	CTA	GCA	GGG	GAA	AGA	2922
	Ser	Ser	Thr	Val	Ala	Ile	Asp	His	Ser	Leu	Ser	Leu	Ala	Gly	Glu	Arg	
			330				335					340					
15	ACT	TGG	GCT	GAA	ACA	ATG	GGT	TTA	AAT	ACC	GCT	GAT	ACA	GCA	AGA	TTA	2970
	Thr	Trp	Ala	Glu	Thr		Gly	Leu	Asn	Thr		Asp	Thr	Ala	Arg	Leu	
						350					355					360	
	AAT	GCC	AAT	ATT	AGA	TAT	GTA	AAT	ACT	GGG	ACG	GCT	CCA	ATC	TAC	AAC	3018
	Asn	Ala	Asn	Ile	Arg	Tyr	Val	Asn	Thr	Gly	Thr	Ala	Pro	Ile	Tyr	Asn	
					365					370					375		
20	GTG	TTA	CCA	ACG	ACT	TCG	TTA	GTG	TTA	GGA	AAA	AAT	CAA	ACA	CTC	GCG	3066
	Val	Leu	Pro	Thr	Thr	Ser	Leu	Val	Leu	Gly	Lys	Asn	Gln	Thr	Leu	Ala	
				380					385					390			
25	ACA	ATT	AAA	GCT	AAG	GAA	AAC	CAA	TTA	AGT	CAA	ATA	CTT	GCA	CCT	AAT	3114
	Thr	Ile	Lys	Ala	Lys	Glu	Asn	Gln	Leu	Ser	Gln	Ile	Leu	Ala	Pro	Asn	
			395					400					405				
30	AAT	TAT	TAT	CCT	TCT	AAA	AAC	TTG	GCG	CCA	ATC	GCA	TTA	AAT	GCA	CAA	3162
	Asn	Tyr	Tyr	Pro	Ser	Lys	Asn	Leu	Ala	Pro	Ile	Ala	Leu	Asn	Ala	Gln	
			410				415					420					
35	GAC	GAT	TTC	AGT	TCT	ACT	CCA	ATT	ACA	ATG	AAT	TAC	AAT	CAA	TTT	CTT	3210
	Asp	Asp	Phe	Ser	Ser	Thr	Pro	Ile	Thr	Met	Asn	Tyr	Asn	Gln	Phe	Leu	
						430					435					440	
	GAG	TTA	GAA	AAA	ACG	AAA	CAA	TTA	AGA	TTA	GAT	ACG	GAT	CAA	GTA	TAT	3258
	Glu	Leu	Glu	Lys	Thr	Lys	Gln	Leu	Arg	Leu	Asp	Thr	Asp	Gln	Val	Tyr	
					445					450					455		
40	GGG	AAT	ATA	GCA	ACA	TAC	AAT	TTT	GAA	AAT	GGA	AGA	GTG	AGG	GTG	GAT	3306
	Gly	Asn	Ile	Ala	Thr	Tyr	Asn	Phe	Glu	Asn	Gly	Arg	Val	Arg	Val	Asp	
				460					465					470			
45	ACA	GGC	TCG	AAC	TGG	AGT	GAA	GTG	TTA	CCG	CAA	ATT	CAA	GAA	ACA	ACT	3354
	Thr	Gly	Ser	Asn	Trp	Ser	Glu	Val	Leu	Pro	Gln	Ile	Gln	Glu	Thr	Thr	
				475				480					485				
50	GCA	CGT	ATC	ATT	TTT	AAT	GGA	AAA	GAT	TTA	AAT	CTG	GTA	GAA	AGG	CGG	3402
	Ala	Arg	Ile	Ile	Phe	Asn	Gly	Lys	Asp	Leu	Asn	Leu	Val	Glu	Arg	Arg	
			490				495					500					
55	ATA	GCG	GCG	GTT	AAT	CCT	AGT	GAT	CCA	TTA	GAA	ACG	ACT	AAA	CCG	GAT	3450
	Ile	Ala	Ala	Val	Asn	Pro	Ser	Asp	Pro	Leu	Glu	Thr	Thr	Lys	Pro	Asp	
						510					515					520	
	ATG	ACA	TTA	AAA	GAA	GCC	CTT	AAA	ATA	GCA	TTT	GGA	TTT	AAC	GAA	CCG	3498
	Met	Thr	Leu	Lys	Glu	Ala	Leu	Lys	Ile	Ala	Phe	Gly	Phe	Asn	Glu	Pro	
					525					530					535		
60	AAT	GGA	AAC	TTA	CAA	TAT	CAA	GCG	AAA	GAC	ATA	ACC	GAA	TTT	GAT	TTT	3546
	Asn	Gly	Asn	Leu	Gln	Tyr	Gln	Gly	Lys	Asp	Ile	Thr	Glu	Phe	Asp	Phe	
				540				545						550			
65	AAT	TTC	GAT	CAA	CAA	ACA	TCT	CAA	AAT	ATC	AAG	AAT	CAG	TTA	GCG	GAA	3594
	Asn	Phe	Asp	Gln	Gln	Thr	Ser	Gln	Asn	Ile	Lys	Asn	Gln	Leu	Ala	Glu	
			555					560					565				
	TTA	AAC	GCA	ACT	AAC	ATA	TAT	ACT	GTA	TTA	GAT	AAA	ATC	AAA	TTA	AAT	3642
	Leu	Asn	Ala	Thr	Asn	Ile	Tyr	Thr	Val	Leu	Asp	Lys	Ile	Lys	Leu	Asn	

	570	575	580	
5	GCA AAA ATG AAT ATT TTA ATA AGA GAT AAA CGT TTT CAT TAT GAT AGA Ala Lys Met Asn Ile Leu Ile Arg Asp Lys Arg Phe His Tyr Asp Arg 585 590 595 600			3690
10	AAT AAC ATA GCA GTT GGG GCG GAT GAG TCA GTA GTT AAG GAG GCT CAT Asn Asn Ile Ala Val Gly Ala Asp Glu Ser Val Val Lys Glu Ala His 605 610 615			3738
15	AGA GAA GTA ATT AAT TCG TCA ACA GAG GGA TTA TTG TTA AAT ATT GAT Arg Glu Val Ile Asn Ser Ser Thr Glu Gly Leu Leu Leu Asn Ile Asp 620 625 630			3786
20	AAG GAT ATA AGA AAA ATA TTA TCA GGT TAT ATT GTA GAA ATT GAA GAT Lys Asp Ile Arg Lys Ile Leu Ser Gly Tyr Ile Val Glu Ile Glu Asp 635 640 645			3834
25	ACT GAA GGG CTT AAA GAA GTT ATA AAT GAC AGA TAT GAT ATG TTG AAT Thr Glu Gly Leu Lys Glu Val Ile Asn Asp Arg Tyr Asp Met Leu Asn 650 655 660			3882
30	ATT TCT AGT TTA CGG CAA GAT GGA AAA ACA TTT ATA GAT TTT AAA AAA Ile Ser Ser Leu Arg Gln Asp Gly Lys Thr Phe Ile Asp Phe Lys Lys 665 670 675 680			3930
35	TAT AAT GAT AAA TTA CCG TTA TAT ATA AGT AAT CCC AAT TAT AAG GTA Tyr Asn Asp Lys Leu Pro Leu Tyr Ile Ser Asn Pro Asn Tyr Lys Val 685 690 695			3978
40	AAT GTA TAT GCT GTT ACT AAA GAA AAC ACT ATT ATT AAT CCT AGT GAG Asn Val Tyr Ala Val Thr Lys Glu Asn Thr Ile Ile Asn Pro Ser Glu 700 705 710			4026
45	AAT GGG GAT ACT AGT ACC AAC GGG ATC AAG AAA ATT TTA ATC TTT TCT Asn Gly Asp Thr Ser Thr Asn Gly Ile Lys Lys Ile Leu Ile Phe Ser 715 720 725			4074
50	AAA AAA GGC TAT GAG ATA GGA TAAGGTAATT CTAGGTGATT TTAAATTAT Lys Lys Gly Tyr Glu Ile Gly 730 735			4125
55	CTAAAAACA GTAAAATTAA AACATACTCT TTTTGTAAGA AATACAAGGA GAGTATGTTT			4185
60	TAAACAGTAA TCTAAATCAT CATAATCCTT TGAGATTGTT TGTAGGATCC			4235

(2) INFORMATION FOR SEQ ID NO:4:

- (i) SEQUENCE CHARACTERISTICS:
 (A) LENGTH: 735 amino acids
 (B) TYPE: amino acid
 (D) TOPOLOGY: linear

- (ii) MOLECULE TYPE: protein

(xi) SEQUENCE DESCRIPTION SEQ ID NO 4:

60	Glu Val Lys Gln Glu Asn Arg Leu Leu Asn Glu Ser Glu Ser Ser Ser 1 5 10 15
	Gln Gly Leu Leu Gly Tyr Tyr Phe Ser Asp Leu Asn Phe Gln Ala Pro 20 25 30
65	Met Val Val Thr Ser Ser Thr Thr Gly Asp Leu Ser Ile Pro Ser Ser 35 40 45
	Glu Leu Glu Asn Ile Pro Ser Glu Asn Gln Tyr Phe Gln Ser Ala Ile 50 55 60

Trp Ser Gly Phe Ile Lys Val Lys Lys Ser Asp Glu Tyr Thr Phe Ala
 65 70 75 80
 5 Thr Ser Ala Asp Asn His Val Thr Met Trp Val Asp Asp Gln Glu Val
 85 90 95
 Ile Asn Lys Ala Ser Asn Ser Asn Lys Ile Arg Leu Glu Lys Gly Arg
 100 105 110
 10 Leu Tyr Gln Ile Lys Ile Gln Tyr Gln Arg Glu Asn Pro Thr Glu Lys
 115 120 125
 Gly Leu Asp Phe Lys Leu Tyr Trp Thr Asp Ser Gln Asn Lys Lys Glu
 130 135 140
 15 Val Ile Ser Ser Asp Asn Leu Gln Leu Pro Glu Leu Lys Gln Lys Ser
 145 150 155 160
 Ser Asn Ser Arg Lys Lys Arg Ser Thr Ser Ala Gly Pro Thr Val Pro
 165 170 175
 Asp Arg Asp Asn Asp Gly Ile Pro Asp Ser Leu Glu Val Glu Gly Tyr
 180 185 190
 25 Thr Val Asp Val Lys Asn Lys Arg Thr Phe Leu Ser Pro Trp Ile Ser
 195 200 205
 Asn Ile His Glu Lys Lys Gly Leu Thr Lys Tyr Lys Ser Ser Pro Glu
 210 215 220
 30 Lys Trp Ser Thr Ala Ser Asp Pro Tyr Ser Asp Phe Glu Lys Val Thr
 225 230 235 240
 Gly Arg Ile Asp Lys Asn Val Ser Pro Glu Ala Arg His Pro Leu Val
 245 250 255
 35 Ala Ala Tyr Pro Ile Val His Val Asp Met Glu Asn Ile Ile Leu Ser
 260 265 270
 40 Lys Asn Glu Asp Gln Ser Thr Gln Asn Thr Asp Ser Glu Thr Arg Thr
 275 280 285
 Ile Ser Lys Asn Thr Ser Thr Ser Arg Thr His Thr Ser Glu Val His
 290 295 300
 45 Gly Asn Ala Glu Val His Ala Ser Phe Phe Asp Ile Gly Gly Ser Val
 305 310 315 320
 Ser Ala Gly Phe Ser Asn Ser Asn Ser Ser Thr Val Ala Ile Asp His
 325 330 335
 50 Ser Leu Ser Leu Ala Gly Glu Arg Thr Trp Ala Glu Thr Met Gly Leu
 340 345 350
 55 Asn Thr Ala Asp Thr Ala Arg Leu Asn Ala Asn Ile Arg Tyr Val Asn
 355 360 365
 Thr Gly Thr Ala Pro Ile Tyr Asn Val Leu Pro Thr Thr Ser Leu Val
 370 375 380
 60 Leu Gly Lys Asn Gln Thr Leu Ala Thr Ile Lys Ala Lys Glu Asn Gln
 385 390 395 400
 Leu Ser Gln Ile Leu Ala Pro Asn Asn Tyr Tyr Pro Ser Lys Asn Leu
 405 410 415
 65 Ala Pro Ile Ala Leu Asn Ala Gln Asp Asp Phe Ser Ser Thr Pro Ile
 420 425 430

83

Thr Met Asn Tyr Asn Gln Phe Leu Glu Leu Glu Lys Thr Lys Gln Leu
 435 440 445
 5 Arg Leu Asp Thr Asp Gln Val Tyr Gly Asn Ile Ala Thr Tyr Asn Phe
 450 455 460
 Glu Asn Gly Arg Val Arg Val Asp Thr Gly Ser Asn Trp Ser Glu Val
 465 470 475 480
 10 Leu Pro Gln Ile Gln Glu Thr Thr Ala Arg Ile Ile Phe Asn Gly Lys
 485 490 495
 Asp Leu Asn Leu Val Glu Arg Arg Ile Ala Ala Val Asn Pro Ser Asp
 500 505 510
 15 Pro Leu Glu Thr Thr Lys Pro Asp Met Thr Leu Lys Glu Ala Leu Lys
 515 520 525
 20 Ile Ala Phe Gly Phe Asn Glu Pro Asn Gly Asn Leu Gln Tyr Gln Gly
 530 535 540
 Lys Asp Ile Thr Glu Phe Asp Phe Asn Phe Asp Gln Gln Thr Ser Gln
 545 550 555 560
 25 Asn Ile Lys Asn Gln Leu Ala Glu Leu Asn Ala Thr Asn Ile Tyr Thr
 565 570 575
 Val Leu Asp Lys Ile Lys Leu Asn Ala Lys Met Asn Ile Leu Ile Arg
 580 585 590
 30 Asp Lys Arg Phe His Tyr Asp Arg Asn Asn Ile Ala Val Gly Ala Asp
 595 600 605
 35 Glu Ser Val Val Lys Glu Ala His Arg Glu Val Ile Asn Ser Ser Thr
 610 615 620
 Glu Gly Leu Leu Leu Asn Ile Asp Lys Asp Ile Arg Lys Ile Leu Ser
 625 630 635 640
 40 Gly Tyr Ile Val Glu Ile Glu Asp Thr Glu Gly Leu Lys Glu Val Ile
 645 650 655
 Asn Asp Arg Tyr Asp Met Leu Asn Ile Ser Ser Leu Arg Gln Asp Gly
 660 665 670
 45 Lys Thr Phe Ile Asp Phe Lys Lys Tyr Asn Asp Lys Leu Pro Leu Tyr
 675 680 685
 50 Ile Ser Asn Pro Asn Tyr Lys Val Asn Val Tyr Ala Val Thr Lys Glu
 690 695 700
 Asn Thr Ile Ile Asn Pro Ser Glu Asn Gly Asp Thr Ser Thr Asn Gly
 705 710 715 720
 55 Ile Lys Lys Ile Leu Ile Phe Ser Lys Lys Gly Tyr Glu Ile Gly
 725 730 735

(2) INFORMATION FOR SEQ ID NO:5:

- 60 (i) SEQUENCE CHARACTERISTICS:
 (A) LENGTH: 1368 base pairs
 (B) TYPE: nucleic acid
 (C) STRANDEDNESS: single
 (D) TOPOLOGY: linear

- 65 (ii) MOLECULE TYPE: DNA (genomic)

(iii) HYPOTHETICAL: NO

(iv) ANTI-SENSE: NO

(vi) ORIGINAL SOURCE:

(A) ORGANISM: *Bacillus anthracis*

(ix) FEATURE:

(A) NAME/KEY: CDS

(B) LOCATION: 1..1368

(D) OTHER INFORMATION: /product=

"LF(1-254)--TR--PE(401-602)"

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:5:

15	GCG GGC GGT CAT GGT GAT GTA GGT ATG CAC GTA AAA GAG AAA GAG AAA Ala Gly Gly His Gly Asp Val Gly Met His Val Lys Glu Lys Glu Lys 1 5 10 15	48
20	AAT AAA GAT GAG AAT AAG AGA AAA GAT GAA GAA CGA AAT AAA ACA CAG Asn Lys Asp Glu Asn Lys Arg Lys Asp Glu Glu Arg Asn Lys Thr Gln 20 25 30	96
25	GAA GAG CAT TTA AAG GAA ATC ATG AAA CAC ATT GTA AAA ATA GAA GTA Glu Glu His Leu Lys Glu Ile Met Lys His Ile Val Lys Ile Glu Val 35 40 45	144
30	AAA GGG GAG GAA GCT GTT AAA AAA GAG GCA GCA GAA AAG CTA CTT GAG Lys Gly Glu Glu Ala Val Lys Lys Glu Ala Ala Glu Lys Leu Leu Glu 50 55 60	192
35	AAA GTA CCA TCT GAT GTT TTA GAG ATG TAT AAA GCA ATT GGA GGA AAG Lys Val Pro Ser Asp Val Leu Glu Met Tyr Lys Ala Ile Gly Gly Lys 65 70 75 80	240
40	ATA TAT ATT GTG GAT GGT GAT ATT ACA AAA CAT ATA TCT TTA GAA GCA Ile Tyr Ile Val Asp Gly Asp Ile Thr Lys His Ile Ser Leu Glu Ala 85 90 95	288
45	TTA TCT GAA GAT AAG AAA AAA ATA AAA GAC ATT TAT GGG AAA GAT GCT Leu Ser Glu Asp Lys Lys Lys Ile Lys Asp Ile Tyr Gly Lys Asp Ala 100 105 110	336
50	TTA TTA CAT GAA CAT TAT GTA TAT GCA AAA GAA GGA TAT GAA CCC GTA Leu Leu His Glu His Tyr Val Tyr Ala Lys Glu Gly Tyr Glu Pro Val 115 120 125	384
55	CTT GTA ATC CAA TCT TCG GAA GAT TAT GTA GAA AAT ACT GAA AAG GCA Leu Val Ile Gln Ser Ser Glu Asp Tyr Val Glu Asn Thr Glu Lys Ala 130 135 140	432
	CTG AAC GTT TAT TAT GAA ATA GGT AAG ATA TTA TCA AGG GAT ATT TTA Leu Asn Val Tyr Tyr Glu Ile Gly Lys Ile Leu Ser Arg Asp Ile Leu 145 150 155 160	480

85

	AGT AAA ATT AAT CAA CCA TAT CAG AAA TTT TTA GAT GTA TTA AAT ACC	528
	Ser Lys Ile Asn Gln Pro Tyr Gln Lys Phe Leu Asp Val Leu Asn Thr	
	165 170 175	
5	ATT AAA AAT GCA TCT GAT TCA GAT GGA CAA GAT CTT TTA TTT ACT AAT	576
	Ile Lys Asn Ala Ser Asp Ser Asp Gly Gln Asp Leu Leu Phe Thr Asn	
	180 185 190	
10	CAG CTT AAG GAA CAT CCC ACA GAC TTT TCT GTA GAA TTC TTG GAA CAA	624
	Gln Leu Lys Glu His Pro Thr Asp Phe Ser Val Glu Phe Leu Glu Gln	
	195 200 205	
15	AAT AGC AAT GAG GTA CAA GAA GTA TTT GCG AAA GCT TTT GCA TAT TAT	672
	Asn Ser Asn Glu Val Gln Glu Val Phe Ala Lys Ala Phe Ala Tyr Tyr	
	210 215 220	
20	ATC GAG CCA CAG CAT CGT GAT GTT TTA CAG CTT TAT GCA CCG GAA GCT	720
	Ile Glu Pro Gln His Arg Asp Val Leu Gln Leu Tyr Ala Pro Glu Ala	
	225 230 235 240	
25	TTT AAT TAC ATG GAT AAA TTT AAC GAA CAA GAA ATA AAT CTA CTC GGC	768
	Phe Asn Tyr Met Asp Lys Phe Asn Glu Gln Glu Ile Asn Leu Leu Gly	
	245 250 255	
30	GAC GGC GGC GAC GTC AGC TTC AGC ACC CGC GGC ACG CAG AAC TGG ACG	816
	Asp Gly Gly Asp Val Ser Phe Ser Thr Arg Gly Thr Gln Asn Trp Thr	
	260 265 270	
35	GTG GAG CGG CTG CTC CAG GCG CAC CGC CAA CTG GAG GAG CGC GGC TAT	864
	Val Glu Arg Leu Leu Gln Ala His Arg Gln Leu Glu Glu Arg Gly Tyr	
	275 280 285	
40	GTG TTC GTC GGC TAC CAC GGC ACC TTC CTC GAA GCG GCG CAA AGC ATC	912
	Val Phe Val Gly Tyr His Gly Thr Phe Leu Glu Ala Ala Gln Ser Ile	
	290 295 300	
45	GTC TTC GGC GGG GTG CGC GCG CGC AGC CAG GAC CTC GAC GCG ATC TGG	960
	Val Phe Gly Gly Val Arg Ala Arg Ser Gln Asp Leu Asp Ala Ile Trp	
	305 310 315 320	
50	CGC GGT TTC TAT ATC GCC GGC GAT CCG GCG CTG GCC TAC GGC TAC GCC	1008
	Arg Gly Phe Tyr Ile Ala Gly Asp Pro Ala Leu Ala Tyr Gly Tyr Ala	
	325 330 335	
55	CAG GAC CAG GAA CCC GAC GCA CGC GGC CGG ATC CGC AAC GGT GCC CTG	1056
	Gln Asp Gln Glu Pro Asp Ala Arg Gly Arg Ile Arg Asn Gly Ala Leu	
	340 345 350	
60	CTG CGG GTC TAT GTG CCG CGC TCG AGC CTG CCG GGC TTC TAC CGC ACC	1104
	Leu Arg Val Tyr Val Pro Arg Ser Ser Leu Pro Gly Phe Tyr Arg Thr	
	355 360 365	
65	AGC CTG ACC CTG GCC GCG CCG GAG GCG GCG GGC GAG GTC GAA CGG CTG	1152
	Ser Leu Thr Leu Ala Ala Pro Glu Ala Ala Gly Glu Val Glu Arg Leu	
	370 375 380	
70	ATC GGC CAT CCG CTG CCG CTG CGC CTG GAC GCC ATC ACC GGC CCC GAG	1200
	Ile Gly His Pro Leu Pro Leu Arg Leu Asp Ala Ile Thr Gly Pro Glu	
	385 390 395 400	
75	GAG GAA GGC GGG CGC CTG GAG ACC ATT CTC GGC TGG CCG CTG GCC GAG	1248
	Glu Glu Gly Gly Arg Leu Glu Thr Ile Leu Gly Trp Pro Leu Ala Glu	
	405 410 415	
80	CGC ACC GTG GTG ATT CCC TCG GCG ATC CCC ACC GAC CCG CGC AAC GTC	1296
	Arg Thr Val Val Ile Pro Ser Ala Ile Pro Thr Asp Pro Arg Asn Val	
	420 425 430	
85	GGC GGC GAC CTC GAC CCG TCC AGC ATC CCC GAC AAG GAA CAG GCG ATC	1344

86

Gly Gly Asp Leu Asp Pro Ser Ser Ile Pro Asp Lys Glu Gln Ala Ile
 435 440 445

5 AGC GCC CTG CCG GAC TAC GCC AGC 1368
 Ser Ala Leu Pro Asp Tyr Ala Ser
 450 455

(2) INFORMATION FOR SEQ ID NO:6:

10

- (i) SEQUENCE CHARACTERISTICS:
 (A) LENGTH: 456 amino acids
 (B) TYPE: amino acid
 (D) TOPOLOGY: linear

15

(ii) MOLECULE TYPE: protein

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:6:

20

Ala Gly Gly His Gly Asp Val Gly Met His Val Lys Glu Lys Glu Lys
 1 5 10 15

25

Asn Lys Asp Glu Asn Lys Arg Lys Asp Glu Glu Arg Asn Lys Thr Gln
 20 25 30

Glu Glu His Leu Lys Glu Ile Met Lys His Ile Val Lys Ile Glu Val
 35 40 45

30

Lys Gly Glu Glu Ala Val Lys Lys Glu Ala Ala Glu Lys Leu Leu Glu
 50 55 60

Lys Val Pro Ser Asp Val Leu Glu Met Tyr Lys Ala Ile Gly Gly Lys
 65 70 75 80

35

Ile Tyr Ile Val Asp Gly Asp Ile Thr Lys His Ile Ser Leu Glu Ala
 85 90 95

Leu Ser Glu Asp Lys Lys Lys Ile Lys Asp Ile Tyr Gly Lys Asp Ala
 100 105 110

40

Leu Leu His Glu His Tyr Val Tyr Ala Lys Glu Gly Tyr Glu Pro Val
 115 120 125

45

Leu Val Ile Gln Ser Ser Glu Asp Tyr Val Glu Asn Thr Glu Lys Ala
 130 135 140

Leu Asn Val Tyr Tyr Glu Ile Gly Lys Ile Leu Ser Arg Asp Ile Leu
 145 150 155 160

50

Ser Lys Ile Asn Gln Pro Tyr Gln Lys Phe Leu Asp Val Leu Asn Thr
 165 170 175

Ile Lys Asn Ala Ser Asp Ser Asp Gly Gln Asp Leu Leu Phe Thr Asn
 180 185 190

55

Gln Leu Lys Glu His Pro Thr Asp Phe Ser Val Glu Phe Leu Glu Gln
 195 200 205

60

Asn Ser Asn Glu Val Gln Glu Val Phe Ala Lys Ala Phe Ala Tyr Tyr
 210 215 220

Ile Glu Pro Gln His Arg Asp Val Leu Gln Leu Tyr Ala Pro Glu Ala
 225 230 235 240

65

Phe Asn Tyr Met Asp Lys Phe Asn Glu Gln Glu Ile Asn Leu Leu Gly
 245 250 255

Asp Gly Gly Asp Val Ser Phe Ser Thr Arg Gly Thr Gln Asn Trp Thr
 260 265 270

87

Val Glu Arg Leu Leu Gln Ala His Arg Gln Leu Glu Glu Arg Gly Tyr
275 280 285

5 Val Phe Val Gly Tyr His Gly Thr Phe Leu Glu Ala Ala Gln Ser Ile
290 295 300

Val Phe Gly Gly Val Arg Ala Arg Ser Gln Asp Leu Asp Ala Ile Trp
305 310 315 320

10 Arg Gly Phe Tyr Ile Ala Gly Asp Pro Ala Leu Ala Tyr Gly Tyr Ala
325 330 335

Gln Asp Gln Glu Pro Asp Ala Arg Gly Arg Ile Arg Asn Gly Ala Leu
340 345 350

15 Leu Arg Val Tyr Val Pro Arg Ser Ser Leu Pro Gly Phe Tyr Arg Thr
355 360 365

20 Ser Leu Thr Leu Ala Ala Pro Glu Ala Ala Gly Glu Val Glu Arg Leu
370 375 380

Ile Gly His Pro Leu Pro Leu Arg Leu Asp Ala Ile Thr Gly Pro Glu
385 390 395 400

25 Glu Glu Gly Gly Arg Leu Glu Thr Ile Leu Gly Trp Pro Leu Ala Glu
405 410 415

Arg Thr Val Val Ile Pro Ser Ala Ile Pro Thr Asp Pro Arg Asn Val
420 425 430

30 Gly Gly Asp Leu Asp Pro Ser Ser Ile Pro Asp Lys Glu Gln Ala Ile
435 440 445

35 Ser Ala Leu Pro Asp Tyr Ala Ser
450 455

(2) INFORMATION FOR SEQ ID NO:7:

(i) SEQUENCE CHARACTERISTICS:
40 (A) LENGTH: 1425 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

45 (ii) MOLECULE TYPE: DNA (genomic)

(iii) HYPOTHETICAL: NO

(vi) ORIGINAL SOURCE:
50 (A) ORGANISM: Bacillus anthracis

(ix) FEATURE:
55 (A) NAME/KEY: CDS
(B) LOCATION: 1..1416
(D) OTHER INFORMATION: /product=
"LF(1-254)--TR--PE(398-613)"

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:7:

60 ATG GTA CCA GCG GGC GGT CAT GGT GAT GTA GGT ATG CAC GTA AAA GAG 48
Met Val Pro Ala Gly Gly His Gly Asp Val Gly Met His Val Lys Glu
1 5 10 15

65 AAA GAG AAA AAT AAA GAT GAG AAT AAG ACA AAA GAT GAA GAA CGA AAT 96
Lys Glu Lys Asn Lys Asp Glu Asn Lys Arg Lys Asp Glu Glu Arg Asn
20 25 30

AAA ACA CAG GAA GAG CAT TTA AAG GAA ATC ATG AAA CAC ATT GTA AAA 144

88

	Lys	Thr	Gln	Glu	Glu	His	Leu	Lys	Glu	Ile	Met	Lys	His	Ile	Val	Lys	
			35					40					45				
5	ATA	GAA	GTA	AAA	GGG	GAG	GAA	GCT	GTT	AAA	AAA	GAG	GCA	GCA	GAA	AAG	192
	Ile	Glu	Val	Lys	Gly	Glu	Glu	Ala	Val	Lys	Lys	Glu	Ala	Ala	Glu	Lys	
		50					55					60					
10	CTA	CTT	GAG	AAA	GTA	CCA	TCT	GAT	GTT	TTA	GAG	ATG	TAT	AAA	GCA	ATT	240
	Leu	Leu	Glu	Lys	Val	Pro	Ser	Asp	Val	Leu	Glu	Met	Tyr	Lys	Ala	Ile	
		65				70					75					80	
15	GGA	GGA	AAG	ATA	TAT	ATT	GTG	GAT	GGT	GAT	ATT	ACA	AAA	CAT	ATA	TCT	288
	Gly	Gly	Lys	Ile	Tyr	Ile	Val	Asp	Gly	Asp	Ile	Thr	Lys	His	Ile	Ser	
					85					90					95		

	TTA GAA GCA TTA TCT GAA GAT AAG AAA AAA ATA AAA GAC ATT TAT GGG Leu Glu Ala Leu Ser Glu Asp Lys Lys Lys Ile Lys Asp Ile Tyr Gly 100 105 110	336
5	AAA GAT GCT TTA TTA CAT GAA CAT TAT GTA TAT GCA AAA GAA GGA TAT Lys Asp Ala Leu Leu His Glu His Tyr Val Tyr Ala Lys Glu Gly Tyr 115 120 125	384
10	GAA CCC GTA CTT GTA ATC CAA TCT TCG GAA GAT TAT GTA GAA AAT ACT Glu Pro Val Leu Val Ile Gln Ser Ser Glu Asp Tyr Val Glu Asn Thr 130 135 140	432
15	GAA AAG GCA CTG AAC GTT TAT TAT GAA ATA GGT AAG ATA TTA TCA AGG Glu Lys Ala Leu Asn Val Tyr Tyr Glu Ile Gly Lys Ile Leu Ser Arg 145 150 155 160	480
20	GAT ATT TTA AGT AAA ATT AAT CAA CCA TAT CAG AAA TTT TTA GAT GTA Asp Ile Leu Ser Lys Ile Asn Gln Pro Tyr Gln Lys Phe Leu Asp Val 165 170 175	528
25	TTA AAT ACC ATT AAA AAT GCA TCT GAT TCA GAT GGA CAA GAT CTT TTA Leu Asn Thr Ile Lys Asn Ala Ser Asp Ser Asp Gly Gln Asp Leu Leu 180 185 190	576
30	TTT ACT AAT CAG CTT AAG GAA CAT CCC ACA GAC TTT TCT GTA GAA TTC Phe Thr Asn Gln Leu Lys Glu His Pro Thr Asp Phe Ser Val Glu Phe 195 200 205	624
35	TTG GAA CAA AAT AGC AAT GAG GTA CAA GAA GTA TTT GCG AAA GCT TTT Leu Glu Gln Asn Ser Asn Glu Val Gln Glu Val Phe Ala Lys Ala Phe 210 215 220	672
40	GCA TAT TAT ATC GAG CCA CAG CAT CGT GAT GTT TTA CAG CTT TAT GCA Ala Tyr Tyr Ile Glu Pro Gln His Arg Asp Val Leu Gln Leu Tyr Ala 225 230 235 240	720
45	CCG GAA GCT TTT AAT TAC ATG GAT AAA TTT AAC GAA CAA GAA ATA AAT Pro Glu Ala Phe Asn Tyr Met Asp Lys Phe Asn Glu Gln Glu Ile Asn 245 250 255	768
50	CTA ACG CGT GCG GAG TTC CTC GGC GAC GGC GGC GAC GTC AGC TTC AGC Leu Thr Arg Ala Glu Phe Leu Gly Asp Gly Gly Asp Val Ser Phe Ser 260 265 270	816
55	ACC CGC GGC ACG CAG AAC TGG ACG GTG GAG CGG CTG CTC CAG GCG CAC Thr Arg Gly Thr Gln Asn Trp Thr Val Glu Arg Leu Leu Gln Ala His 275 280 285	864
60	CGC CAA CTG GAG GAG CGC GGC TAT GTG TTC GTC GGC TAC CAC GGC ACC Arg Gln Leu Glu Glu Arg Gly Tyr Val Phe Val Gly Tyr His Gly Thr 290 295 300	912
65	TTC CTC GAA GCG GCG CAA AGC ATC CTC TTC GGC GGG GTG CGC GCG CGC Phe Leu Glu Ala Ala Gln Ser Ile Val Phe Gly Gly Val Arg Ala Arg 305 310 315 320	960
70	AGC CAG GAC CTC GAC GCG ATC TGG CGC GGT TTC TAT ATC GCG GGC GAT Ser Gln Asp Leu Asp Ala Ile Trp Arg Gly Phe Tyr Ile Ala Gly Asp 325 330 335	1008
75	CCG GCG CTG GCC TAC GGC TAC GCC CAG GAC CAG GAA CCC GAC GCA CGC Pro Ala Leu Ala Tyr Gly Tyr Ala Gln Asp Gln Glu Pro Asp Ala Arg 340 345 350	1056
80	GGC CGG ATC CGC AAC GGT GCC CTG CTG CGG GTC TAT GTG CCG CGC TCG Gly Arg Ile Arg Asn Gly Ala Leu Leu Arg Val Tyr Val Pro Arg Ser 355 360 365	1104
85	AGC CTG CCG GGC TTC TAC CGC ACC AGC CTG ACC CTG GCC GCG CCG GAG	1152

90

	Ser	Leu	Pro	Gly	Phe	Tyr	Arg	Thr	Ser	Leu	Thr	Leu	Ala	Ala	Pro	Glu	
	370						375					380					
5	GCG	GCG	GGC	GAG	GTC	GAA	CGG	CTG	ATC	GGC	CAT	CCG	CTG	CCG	CTG	CGC	1200
	Ala	Ala	Gly	Glu	Val	Glu	Arg	Leu	Ile	Gly	His	Pro	Leu	Pro	Leu	Arg	
	385					390				395						400	
10	CTG	GAC	GCC	ATC	ACC	GGC	CCC	GAG	GAG	GAA	GGC	GGG	CGC	CTG	GAG	ACC	1248
	Leu	Asp	Ala	Ile	Thr	Gly	Pro	Glu	Glu	Glu	Gly	Gly	Arg	Leu	Glu	Thr	
					405					410					415		
15	ATT	CTC	GGC	TGG	CCG	CTG	GCC	GAG	CGC	ACC	GTG	GTG	ATT	CCC	TCG	GCG	1296
	Ile	Leu	Gly	Trp	Pro	Leu	Ala	Glu	Arg	Thr	Val	Val	Ile	Pro	Ser	Ala	
				420					425					430			
20	ATC	CCC	ACC	GAC	CCG	CGC	AAC	GTC	GGC	GGC	GAC	CTC	GAC	CCG	TCC	AGC	1344
	Ile	Pro	Thr	Asp	Pro	Arg	Asn	Val	Gly	Gly	Asp	Leu	Asp	Pro	Ser	Ser	
				435				440					445				
25	ATC	CCC	GAC	AAG	GAA	CAG	GCG	ATC	AGC	GCC	CTG	CCG	GAC	TAC	GCC	AGC	1392
	Ile	Pro	Asp	Lys	Glu	Gln	Ala	Ile	Ser	Ala	Leu	Pro	Asp	Tyr	Ala	Ser	
				450			455					460					
30	CAG	CCC	GGC	AAA	CCG	CCG	CGC	GAG	GACCTGAAG								1425
	Gln	Pro	Gly	Lys	Pro	Pro	Arg	Glu									
	465						470										
	(2) INFORMATION FOR SEQ ID NO:8:																
35	(i) SEQUENCE CHARACTERISTICS:																
	(A) LENGTH: 472 amino acids																
	(B) TYPE: amino acid																
	(D) TOPOLOGY: linear																
40	(ii) MOLECULE TYPE: protein																
	(xi) SEQUENCE DESCRIPTION: SEQ ID NO:8:																
40	Met	Val	Pro	Ala	Gly	Gly	His	Gly	Asp	Val	Gly	Met	His	Val	Lys	Glu	
	1				5				10						15		

Lys Glu Lys Asn Lys Asp Glu Asn Lys Arg Lys Asp Glu Glu Arg Asn
 20 25 30
 5 Lys Thr Gln Glu Glu His Leu Lys Glu Ile Met Lys His Ile Val Lys
 35 40 45
 Ile Glu Val Lys Gly Glu Glu Ala Val Lys Lys Glu Ala Ala Glu Lys
 50 55 60
 10 Leu Leu Glu Lys Val Pro Ser Asp Val Leu Glu Met Tyr Lys Ala Ile
 65 70 75 80
 Gly Gly Lys Ile Tyr Ile Val Asp Gly Asp Ile Thr Lys His Ile Ser
 85 90 95
 15 Leu Glu Ala Leu Ser Glu Asp Lys Lys Lys Ile Lys Asp Ile Tyr Gly
 100 105 110
 20 Lys Asp Ala Leu Leu His Glu His Tyr Val Tyr Ala Lys Glu Gly Tyr
 115 120 125
 Glu Pro Val Leu Val Ile Gln Ser Ser Glu Asp Tyr Val Glu Asn Thr
 130 135 140
 25 Glu Lys Ala Leu Asn Val Tyr Tyr Glu Ile Gly Lys Ile Leu Ser Arg
 145 150 155 160
 Asp Ile Leu Ser Lys Ile Asn Gln Pro Tyr Gln Lys Phe Leu Asp Val
 165 170 175
 30 Leu Asn Thr Ile Lys Asn Ala Ser Asp Ser Asp Gly Gln Asp Leu Leu
 180 185 190
 Phe Thr Asn Gln Leu Lys Glu His Pro Thr Asp Phe Ser Val Glu Phe
 195 200 205
 35 Leu Glu Gln Asn Ser Asn Glu Val Gln Glu Val Phe Ala Lys Ala Phe
 210 215 220
 40 Ala Tyr Tyr Ile Glu Pro Gln His Arg Asp Val Leu Gln Leu Tyr Ala
 225 230 235 240
 Pro Glu Ala Phe Asn Tyr Met Asp Lys Phe Asn Glu Gln Glu Ile Asn
 245 250 255
 45 Leu Thr Arg Ala Glu Phe Leu Gly Asp Gly Gly Asp Val Ser Phe Ser
 260 265 270
 50 Thr Arg Gly Thr Gln Asn Trp Thr Val Glu Arg Leu Leu Gln Ala His
 275 280 285
 Arg Gln Leu Glu Glu Arg Gly Tyr Val Phe Val Gly Tyr His Gly Thr
 290 295 300
 55 Phe Leu Glu Ala Ala Gln Ser Ile Val Phe Gly Gly Val Arg Ala Arg
 305 310 315 320
 Ser Gln Asp Ser Asp Ala Ile Trp Arg Gly Phe Tyr Ile Ala Gly Asp
 325 330 335
 60 Pro Ala Leu Ala Tyr Gly Tyr Ala Gln Asp Gln Glu Pro Asp Ala Arg
 340 345 350
 Gly Arg Ile Arg Asn Gly Ala Leu Leu Arg Val Tyr Val Pro Arg Ser
 355 360 365
 65 Ser Leu Pro Gly Phe Tyr Arg Thr Ser Leu Thr Leu Ala Ala Pro Glu
 370 375 380
 Ala Ala Gly Glu Val Glu Arg Leu Ile Gly His Pro Leu Pro Leu Arg

	385		390		395		400
	Leu Asp Ala Ile Thr Gly Pro Glu Glu Glu Gly Gly Arg Leu Glu Thr		405		410		415
5	Ile Leu Gly Trp Pro Leu Ala Glu Arg Thr Val Val Ile Pro Ser Ala		420		425		430
10	Ile Pro Thr Asp Pro Arg Asn Val Gly Gly Asp Leu Asp Pro Ser Ser		435		440		445
	Ile Pro Asp Lys Glu Gln Ala Ile Ser Ala Leu Pro Asp Tyr Ala Ser		450		455		460
15	Gln Pro Gly Lys Pro Pro Arg Glu		465		470		

(2) INFORMATION FOR SEQ ID NO:9:

- 20 (i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 1524 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear
- 25 (ii) MOLECULE TYPE: DNA (genomic)
- (iii) HYPOTHETICAL: NO
- 30 (vi) ORIGINAL SOURCE:
(A) ORGANISM: Bacillus anthracis
- (ix) FEATURE:
(A) NAME/KEY: CDS
35 (B) LOCATION: 1..1524
(D) OTHER INFORMATION: /product=
"LF(1-254)--TR--PE(362-613)"
- 40 (xi) SEQUENCE DESCRIPTION: SEQ ID NO:9:

	GCG GGC GGT CAT GGT GAT GTA GGT ATG CAC GTA AAA GAG AAA GAG AAA	48
	Ala Gly Gly His Gly Asp Val Gly Met His Val Lys Glu Lys Glu Lys	
	1 5 10 15	
5	AAT AAA GAT GAG AAT AAG AGA AAA GAT GAA GAA CGA AAT AAA ACA CAG	96
	Asn Lys Asp Glu Asn Lys Arg Lys Asp Glu Glu Arg Asn Lys Thr Gln	
	20 25 30	
10	GAA GAG CAT TTA AAG GAA ATC ATG AAA CAC ATT GTA AAA ATA GAA GTA	144
	Glu Glu His Leu Lys Glu Ile Met Lys His Ile Val Lys Ile Glu Val	
	35 40 45	
15	AAA GGG GAG GAA GCT GTT AAA AAA GAG GCA GCA GAA AAG CTA CTT GAG	192
	Lys Gly Glu Glu Ala Val Lys Lys Glu Ala Ala Glu Lys Leu Leu Glu	
	50 55 60	
20	AAA GTA CCA TCT GAT GTT TTA GAG ATG TAT AAA GCA ATT GGA GGA AAG	240
	Lys Val Pro Ser Asp Val Leu Glu Met Tyr Lys Ala Ile Gly Gly Lys	
	65 70 75 80	
25	ATA TAT ATT GTG GAT GGT GAT ATT ACA AAA CAT ATA TCT TTA GAA GCA	288
	Ile Tyr Ile Val Asp Gly Asp Ile Thr Lys His Ile Ser Leu Glu Ala	
	85 90 95	
30	TTA TCT GAA GAT AAG AAA AAA ATA AAA GAC ATT TAT GGG AAA GAT GCT	336
	Leu Ser Glu Asp Lys Lys Lys Ile Lys Asp Ile Tyr Gly Lys Asp Ala	
	100 105 110	
35	TTA TTA CAT GAA CAT TAT GTA TAT GCA AAA GAA GGA TAT GAA CCC GTA	384
	Leu Leu His Glu His Tyr Val Tyr Ala Lys Glu Gly Tyr Glu Pro Val	
	115 120 125	
40	CTT GTA ATC CAA TCT TCG GAA GAT TAT GTA GAA AAT ACT GAA AAG GCA	432
	Leu Val Ile Gln Ser Ser Glu Asp Tyr Val Glu Asn Thr Glu Lys Ala	
	130 135 140	
45	CTG AAC GTT TAT TAT GAA ATA GGT AAG ATA TTA TCA AGG GAT ATT TTA	480
	Leu Asn Val Tyr Tyr Glu Ile Gly Lys Ile Leu Ser Arg Asp Ile Leu	
	145 150 155 160	
50	AGT AAA ATT AAT CAA CCA TAT CAG AAA TTT TTA GAT GTA TTA AAT ACC	528
	Ser Lys Ile Asn Gln Pro Tyr Gln Lys Phe Leu Asp Val Leu Asn Thr	
	165 170 175	
55	ATT AAA AAT GCA TCT GAT TCA GAT GGA CAA GAT CTT TTA TTT ACT AAT	576
	Ile Lys Asn Ala Ser Asp Ser Asp Gly Gln Asp Leu Leu Phe Thr Asn	
	180 185 190	
60	CAG CTT AAG GAA CAT CCC ACA GAC TTT TCT GTA GAA TTC TTG GAA CAA	624
	Gln Leu Lys Glu His Pro Thr Asp Phe Ser Val Glu Phe Leu Glu Gln	
	195 200 205	
65	AAT AGC AAT GAG GTA CAA GAA GTA TTT GCG AAA GCT TTT GCA TAT TAT	672
	Asn Ser Asn Glu Val Gln Glu Val Phe Ala Lys Ala Phe Ala Tyr Tyr	
	210 215 220	
70	ATC GAG CCA CAG CAT CGT GAT GTT TTA CAG CTT TAT GCA CCG GAA GCT	720
	Ile Glu Pro Gln His Arg Asp Val Leu Gln Leu Tyr Ala Pro Glu Ala	
	225 230 235 240	
75	TTT AAT TAC ATG GAT AAA TTT AAC GAA CAA GAA ATA AAT CTA ACG CGT	768
	Phe Asn Tyr Met Asp Lys Phe Asn Glu Gln Glu Ile Asn Leu Thr Arg	
	245 250 255	
80	GCG GCC AAC GCC GAC GTG GTG AGC CTG ACC TGC CCG GTC GCC GCC GGT	816
	Ala Ala Asn Ala Asp Val Val Ser Leu Thr Cys Pro Val Ala Ala Gly	
	260 265 270	
85	GAA TGC GCG GGC CCG GCG GAC AGC GGC GAC GCC CTG CTG GAG CGC AAC	864

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	Glu	Cys	Ala	Gly	Pro	Ala	Asp	Ser	Gly	Asp	Ala	Leu	Leu	Glu	Arg	Asn	
			275					280					285				
5	TAT	CCC	ACT	GGC	GCG	GAG	TTC	CTC	GGC	GAC	GGC	GGC	GAC	GTC	AGC	TTC	912
	Tyr	Pro	Thr	Gly	Ala	Glu	Phe	Leu	Gly	Asp	Gly	Gly	Asp	Val	Ser	Phe	
		290					295					300					
10	AGC	ACC	CGC	GGC	ACG	CAG	AAC	TGG	ACG	GTG	GAG	CGG	CTG	CTC	CAG	GCG	960
	Ser	Thr	Arg	Gly	Thr	Gln	Asn	Trp	Thr	Val	Glu	Arg	Leu	Leu	Gln	Ala	
	305					310					315					320	
15	CAC	CGC	CAA	CTG	GAG	GAG	CGC	GGC	TAT	GTG	TTC	GTC	GGC	TAC	CAC	GGC	1008
	His	Arg	Gln	Leu	Glu	Glu	Arg	Gly	Tyr	Val	Phe	Val	Gly	Tyr	His	Gly	
				325					330						335		
20	ACC	TTC	CTC	GAA	GCG	GCG	CAA	AGC	ATC	GTC	TTC	GGC	GGG	GTG	CGC	GCG	1056
	Thr	Phe	Leu	Glu	Ala	Ala	Gln	Ser	Ile	Val	Phe	Gly	Gly	Val	Arg	Ala	
				340					345					350			
25	CGC	AGC	CAG	GAC	CTC	GAC	GCG	ATC	TGG	CGC	GGT	TTC	TAT	ATC	GCC	GGC	1104
	Arg	Ser	Gln	Asp	Leu	Asp	Ala	Ile	Trp	Arg	Gly	Phe	Tyr	Ile	Ala	Gly	
			355					360					365				
30	GAT	CCG	GCG	CTG	GCC	TAC	GGC	TAC	GCC	CAG	GAC	CAG	GAA	CCC	GAC	GCA	1152
	Asp	Pro	Ala	Leu	Ala	Tyr	Gly	Tyr	Ala	Gln	Asp	Gln	Glu	Pro	Asp	Ala	
		370				375						380					
35	CGC	GGC	CGG	ATC	CGC	AAC	GGT	GCC	CTG	CTG	CGG	GTC	TAT	GTG	CCG	CGC	1200
	Arg	Gly	Arg	Ile	Arg	Asn	Gly	Ala	Leu	Leu	Arg	Val	Tyr	Val	Pro	Arg	
	385					390					395					400	
40	TCG	AGC	CTG	CCG	GGC	TTC	TAC	CGC	ACC	AGC	CTG	ACC	CTG	GCC	GCG	CCG	1248
	Ser	Ser	Leu	Pro	Gly	Phe	Tyr	Arg	Thr	Ser	Leu	Thr	Leu	Ala	Ala	Pro	
				405					410					415			
45	GAG	GCG	GCG	GGC	GAG	GTC	GAA	CGG	CTG	ATC	GGC	CAT	CCG	CTG	CCG	CTG	1296
	Glu	Ala	Ala	Gly	Glu	Val	Glu	Arg	Leu	Ile	Gly	His	Pro	Leu	Pro	Leu	
				420					425				430				
50	CGC	CTG	GAC	GCC	ATC	ACC	GGC	CCC	GAG	GAG	GAA	GGC	GGG	CGC	CTG	GAG	1344
	Arg	Leu	Asp	Ala	Ile	Thr	Gly	Pro	Glu	Glu	Glu	Gly	Gly	Arg	Leu	Glu	
			435					440					445				
55	ACC	ATT	CTC	GGC	TGG	CCG	CTG	GCC	GAG	CGC	ACC	GTG	GTG	ATT	CCC	TCG	1392
	Thr	Ile	Leu	Gly	Trp	Pro	Leu	Ala	Glu	Arg	Thr	Val	Val	Ile	Pro	Ser	
		450					455					460					
60	GCG	ATC	CCC	ACC	GAC	CCG	CGC	AAC	GTC	GGC	GGC	GAC	CTC	GAC	CCG	TCC	1440
	Ala	Ile	Pro	Thr	Asp	Pro	Arg	Asn	Val	Gly	Gly	Asp	Leu	Asp	Pro	Ser	
	465					470					475					480	
65	AGC	ATC	CCC	GAC	AAG	GAA	CAG	GCG	ATC	AGC	GCC	CTG	CCG	GAC	TAC	GCC	1488
	Ser	Ile	Pro	Asp	Lys	Glu	Gln	Ala	Ile	Ser	Ala	Leu	Pro	Asp	Tyr	Ala	
				485						490					495		
70	AGC	CAG	CCC	GGC	AAA	CCG	CCG	CGC	GAG	GAC	CTG	AAG					1524
	Ser	Gln	Pro	Gly	Lys	Pro	Pro	Arg	Glu	Asp	Leu	Lys					
				500					505								

(2) INFORMATION FOR SEQ ID NO:10:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 508 amino acids

(B) TYPE: amino acid

(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: protein

95

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:10:

	Ala	Gly	Gly	His	Gly	Asp	Val	Gly	Met	His	Val	Lys	Glu	Lys	Glu	Lys	1	5	10	15
5	Asn	Lys	Asp	Glu	Asn	Lys	Arg	Lys	Asp	Glu	Glu	Arg	Asn	Lys	Thr	Gln	20	25	30	
10	Glu	Glu	His	Leu	Lys	Glu	Ile	Met	Lys	His	Ile	Val	Lys	Ile	Glu	Val	35	40	45	
	Lys	Gly	Glu	Glu	Ala	Val	Lys	Lys	Glu	Ala	Ala	Glu	Lys	Leu	Leu	Glu	50	55	60	
15	Lys	Val	Pro	Ser	Asp	Val	Leu	Glu	Met	Tyr	Lys	Ala	Ile	Gly	Gly	Lys	65	70	75	80
	Ile	Tyr	Ile	Val	Asp	Gly	Asp	Ile	Thr	Lys	His	Ile	Ser	Leu	Glu	Ala	85	90	95	
20	Leu	Ser	Glu	Asp	Lys	Lys	Lys	Ile	Lys	Asp	Ile	Tyr	Gly	Lys	Asp	Ala	100	105	110	
25	Leu	Leu	His	Glu	His	Tyr	Val	Tyr	Ala	Lys	Glu	Gly	Tyr	Glu	Pro	Val	115	120	125	
30	Leu	Val	Ile	Gln	Ser	Ser	Glu	Asp	Tyr	Val	Glu	Asn	Thr	Glu	Lys	Ala	130	135	140	

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Leu Asn Val Tyr Tyr Glu Ile Gly Lys Ile Leu Ser Arg Asp Ile Leu
 145 150 155 160
 5 Ser Lys Ile Asn Gln Pro Tyr Gln Lys Phe Leu Asp Val Leu Asn Thr
 165 170 175
 Ile Lys Asn Ala Ser Asp Ser Asp Gly Gln Asp Leu Leu Phe Thr Asn
 180 185 190
 10 Gln Leu Lys Glu His Pro Thr Asp Phe Ser Val Glu Phe Leu Glu Gln
 195 200 205
 Asn Ser Asn Glu Val Gln Glu Val Phe Ala Lys Ala Phe Ala Tyr Tyr
 210 215 220
 15 Ile Glu Pro Gln His Arg Asp Val Leu Gln Leu Tyr Ala Pro Glu Ala
 225 230 235 240
 Phe Asn Tyr Met Asp Lys Phe Asn Glu Gln Glu Ile Asn Leu Thr Arg
 245 250 255
 20 Ala Ala Asn Ala Asp Val Val Ser Leu Thr Cys Pro Val Ala Ala Gly
 260 265 270
 25 Glu Cys Ala Gly Pro Ala Asp Ser Gly Asp Ala Leu Leu Glu Arg Asn
 275 280 285
 Tyr Pro Thr Gly Ala Glu Phe Leu Gly Asp Gly Gly Asp Val Ser Phe
 290 295 300
 30 Ser Thr Arg Gly Thr Gln Asn Trp Thr Val Glu Arg Leu Leu Gln Ala
 305 310 315 320
 His Arg Gln Leu Glu Glu Arg Gly Tyr Val Phe Val Gly Tyr His Gly
 325 330 335
 35 Thr Phe Leu Glu Ala Ala Gln Ser Ile Val Phe Gly Gly Val Arg Ala
 340 345 350
 40 Arg Ser Gln Asp Leu Asp Ala Ile Trp Arg Gly Phe Tyr Ile Ala Gly
 355 360 365
 Asp Pro Ala Leu Ala Tyr Gly Tyr Ala Gln Asp Gln Glu Pro Asp Ala
 370 375 380
 45 Arg Gly Arg Ile Arg Asn Gly Ala Leu Leu Arg Val Tyr Val Pro Arg
 385 390 395 400
 50 Ser Ser Leu Pro Gly Phe Tyr Arg Thr Ser Leu Thr Leu Ala Ala Pro
 405 410 415
 Glu Ala Ala Gly Glu Val Glu Arg Leu Ile Gly His Pro Leu Pro Leu
 420 425 430
 55 Arg Leu Asp Ala Ile Thr Gly Pro Glu Glu Glu Gly Gly Arg Leu Glu
 435 440 445
 Thr Ile Leu Gly Trp Pro Leu Ala Glu Arg Thr Val Val Ile Pro Ser
 450 455 460
 60 Ala Ile Pro Thr Asp Pro Arg Asn Val Gly Gly Asp Leu Asp Pro Ser
 465 470 475 480
 Ser Ile Pro Asp Lys Glu Gln Ala Ile Ser Ala Leu Pro Asp Tyr Ala
 485 490 495
 65 Ser Gln Pro Gly Lys Pro Pro Arg Glu Asp Leu Lys
 500 505

(2) INFORMATION FOR SEQ ID NO:11:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 2709 base pairs
 (B) TYPE: nucleic acid
 (C) STRANDEDNESS: single
 (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(iii) HYPOTHETICAL: NO

(vi) ORIGINAL SOURCE:

- (A) ORGANISM:
- Bacillus anthracis*

(ix) FEATURE:

- (A) NAME/KEY: CDS
 (B) LOCATION: 1..2709
 (D) OTHER INFORMATION: /product= "PA(1-725)-----Human CD4 residues(1-178)"

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:11:

GAA GTT AAA CAG GAG AAC CGG TTA TTA AAT GAA TCA GAA TCA AGT TCC	48
Glu Val Lys Gln Glu Asn Arg Leu Leu Asn Glu Ser Glu Ser Ser Ser	
1 5 10 15	
CAG GGG TTA CTA GGA TAC TAT TTT AGT GAT TTG AAT TTT CAA GCA CCC	96
Gln Gly Leu Leu Gly Tyr Tyr Phe Ser Asp Leu Asn Phe Gln Ala Pro	
20 25 30	
ATG GTG GTT ACC TCT TCT ACT ACA GGG GAT TTA TCT ATT CCT AGT TCT	144
Met Val Thr Ser Ser Thr Thr Gly Asp Leu Ser Ile Pro Ser Ser	
35 40 45	
GAG TTA GAA AAT ATT CCA TCG GAA AAC CAA TAT TTT CAA TCT GCT ATT	192
Glu Leu Glu Asn Ile Pro Ser Glu Asn Gln Tyr Phe Gln Ser Ala Ile	
50 55 60	
TGG TCA GGA TTT ATC AAA GTT AAG AAG AGT GAT GAA TAT ACA TTT GCT	240
Trp Ser Gly Phe Ile Lys Val Lys Lys Ser Asp Glu Tyr Thr Phe Ala	
65 70 75 80	
ACT TCC GCT GAT AAT CAT GTA ACA ATG TGG GTA GAT GAC CAA GAA GTG	288
Thr Ser Ala Asp Asn His Val Thr Met Trp Val Asp Asp Gln Glu Val	
85 90 95	
ATT AAT AAA GCT TCT AAT TCT AAC AAA ATC AGA TTA GAA AAA GGA AGA	336
Ile Asn Lys Ala Ser Asn Ser Asn Lys Ile Arg Leu Glu Lys Gly Arg	
100 105 110	
TTA TAT CAA ATA AAA ATT CAA TAT CAA CGA GAA AAT CCT ACT GAA AAA	384
Leu Tyr Gln Ile Lys Ile Gln Tyr Gln Arg Glu Asn Pro Thr Glu Lys	
115 120 125	
GGA TTG GAT TTC AAG TTG TAC TGG ACC GAT TCT CAA AAT AAA AAA GAA	432
Gly Leu Asp Phe Lys Leu Tyr Trp Thr Asp Ser Gln Asn Lys Lys Glu	
130 135 140	
GTG ATT TCT AGT GAT AAC TTA CAA TTG CCA GAA TTA AAA CAA AAA TCT	480
Val Ile Ser Ser Asp Asn Leu Gln Leu Pro Glu Leu Lys Gln Lys Ser	
145 150 155 160	
TCG AAC TCA AGA AAA AAC CCA ACT ACA AGT GCT GGA CCT ATG GTT CCA	528
Ser Asn Ser Arg Lys Lys Arg Ser Thr Ser Ala Gly Pro Thr Val Pro	
165 170 175	
GAC CGT GAC AAT GAT GGA ATC CCT GAT TCA TTA GAG GTA GAA GGA TAT	576
Asp Arg Asp Asn Asp Gly Ile Pro Asp Ser Leu Glu Val Glu Gly Tyr	
180 185 190	

	ACG GTT GAT GTC AAA AAT AAA AGA ACT TTT CTT TCA CCA TGG ATT TCT	624
	Thr Val Asp Val Lys Asn Lys Arg Thr Phe Leu Ser Pro Trp Ile Ser	
	195 200 205	
5	AAT ATT CAT GAA AAG AAA GGA TTA ACC AAA TAT AAA TCA TCT CCT GAA	672
	Asn Ile His Glu Lys Lys Gly Leu Thr Lys Tyr Lys Ser Ser Pro Glu	
	210 215 220	
10	AAA TGG AGC ACG GCT TCT GAT CCG TAC AGT GAT TTC GAA AAG GTT ACA	720
	Lys Trp Ser Thr Ala Ser Asp Pro Tyr Ser Asp Phe Glu Lys Val Thr	
	225 230 235 240	
15	GGA CGG ATT GAT AAG AAT GTA TCA CCA GAG GCA AGA CAC CCC CTT GTG	768
	Gly Arg Ile Asp Lys Asn Val Ser Pro Glu Ala Arg His Pro Leu Val	
	245 250 255	
20	GCA GCT TAT CCG ATT GTA CAT GTA GAT ATG GAG AAT ATT ATT CTC TCA	816
	Ala Ala Tyr Pro Ile Val His Val Asp Met Glu Asn Ile Ile Leu Ser	
	260 265 270	
25	AAA AAT GAG GAT CAA TCC ACA CAG AAT ACT GAT AGT GAA ACG AGA ACA	864
	Lys Asn Glu Asp Gln Ser Thr Gln Asn Thr Asp Ser Glu Thr Arg Thr	
	275 280 285	
30	ATA AGT AAA AAT ACT TCT ACA AGT AGG ACA CAT ACT AGT GAA GTA CAT	912
	Ile Ser Lys Asn Thr Ser Thr Ser Arg Thr His Thr Ser Glu Val His	
	290 295 300	
35	GGA AAT GCA GAA GTG CAT GCG TCG TTC TTT GAT ATT GGT GGG AGT GTA	960
	Gly Asn Ala Glu Val His Ala Ser Phe Phe Asp Ile Gly Gly Ser Val	
	305 310 315 320	
40	TCT GCA GGA TTT AGT AAT TCG AAT TCA AGT ACG GTC GCA ATT GAT CAT	1008
	Ser Ala Gly Phe Ser Asn Ser Asn Ser Ser Thr Val Ala Ile Asp His	
	325 330 335	
45	TCA CTA TCT CTA GCA GGG GAA AGA ACT TGG GCT GAA ACA ATG GGT TTA	1056
	Ser Leu Ser Leu Ala Gly Glu Arg Thr Trp Ala Glu Thr Met Gly Leu	
	340 345 350	
50	AAT ACC GCT GAT ACA GCA AGA TTA AAT GCC AAT ATT AGA TAT GTA AAT	1104
	Asn Thr Ala Asp Thr Ala Arg Leu Asn Ala Asn Ile Arg Tyr Val Asn	
	355 360 365	
55	ACT GGG ACG GCT CCA ATC TAC AAC GTG TTA CCA ACG ACT TCG TTA GTG	1152
	Thr Gly Thr Ala Pro Ile Tyr Asn Val Leu Pro Thr Thr Ser Leu Val	
	370 375 380	
60	TTA GGA AAA AAT CAA ACA CTC GCG ACA ATT AAA GCT AAG GAA AAC CAA	1200
	Leu Gly Lys Asn Gln Thr Leu Ala Thr Ile Lys Ala Lys Glu Asn Gln	
	385 390 395 400	
65	TTA AGT CAA ATA CTT GCA CCT AAT AAT TAT TAT CCT TCT AAA AAC TTG	1248
	Leu Ser Gln Ile Leu Ala Pro Asn Asn Tyr Tyr Pro Ser Lys Asn Leu	
	405 410 415	
70	GCG TCA ATC GCA TTA AAT GCA CAA GAC GAT TTC AGT TCG ACT CCA ATT	1296
	Ala Pro Ile Ala Leu Asn Ala Gln Asp Asp Phe Ser Ser Thr Pro Ile	
	420 425 430	
75	ACA ATG AAT TAC AAT CAA TTT CTT GAG TTA GAA AAA ACG AAA CAA TTA	1344
	Thr Met Asn Tyr Asn Gln Phe Leu Glu Leu Glu Lys Thr Lys Gln Leu	
	435 440 445	
80	AGA TTA GAT ACG GAT CAA GTA TAT GGG AAT ATA GCA ACA TAC AAT TTT	1392
	Arg Leu Asp Thr Asp Gln Val Tyr Gly Asn Ile Ala Thr Tyr Asn Phe	
	450 455 460	
85	GAA AAT GGA AGA GTG AGG GTG GAT ACA GGC TCG AAC TGG AGT GAA GTG	1440

		Glu	Asn	Gly	Arg	Val	Arg	Val	Asp	Thr	Gly	Ser	Asn	Trp	Ser	Glu	Val		
		465					470					475					480		
5		TTA	CCG	CAA	ATT	CAA	GAA	ACA	ACT	GCA	CGT	ATC	ATT	TTT	AAT	GGA	AAA	1488	
		Leu	Pro	Gln	Ile	Gln	Glu	Thr	Thr	Ala	Arg	Ile	Ile	Phe	Asn	Gly	Lys		
						485					490					495			
10		GAT	TTA	AAT	CTG	GTA	GAA	AGG	CGG	ATA	GCG	GCG	GTT	AAT	CCT	AGT	GAT	1536	
		Asp	Leu	Asn	Leu	Val	Glu	Arg	Arg	Ile	Ala	Ala	Val	Asn	Pro	Ser	Asp		
					500					505					510				
15		CCA	TTA	GAA	ACG	ACT	AAA	CCG	GAT	ATG	ACA	TTA	AAA	GAA	GCC	CTT	AAA	1584	
		Pro	Leu	Glu	Thr	Thr	Lys	Pro	Asp	Met	Thr	Leu	Lys	Glu	Ala	Leu	Lys		
				515					520					525					
20		ATA	GCA	TTT	GGA	TTT	AAC	GAA	CCG	AAT	GGA	AAC	TTA	CAA	TAT	CAA	GGG	1632	
		Ile	Ala	Phe	Gly	Phe	Asn	Glu	Pro	Asn	Gly	Asn	Leu	Gln	Tyr	Gln	Gly		
			530					535					540						
25		AAA	GAC	ATA	ACC	GAA	TTT	GAT	TTT	AAT	TTC	GAT	CAA	CAA	ACA	TCT	CAA	1680	
		Lys	Asp	Ile	Thr	Glu	Phe	Asp	Phe	Asn	Phe	Asp	Gln	Gln	Thr	Ser	Gln		
			545				550					555					560		
30		AAT	ATC	AAG	AAT	CAG	TTA	GCG	GAA	TTA	AAC	GCA	ACT	AAC	ATA	TAT	ACT	1728	
		Asn	Ile	Lys	Asn	Gln	Leu	Ala	Glu	Leu	Asn	Ala	Thr	Asn	Ile	Tyr	Thr		
					565						570					575			
35		GTA	TTA	GAT	AAA	ATC	AAA	TTA	AAT	GCA	AAA	ATG	AAT	ATT	TTA	ATA	AGA	1776	
		Val	Leu	Asp	Lys	Ile	Lys	Leu	Asn	Ala	Lys	Met	Asn	Ile	Leu	Ile	Arg		
					580					585					590				
40		GAT	AAA	CGT	TTT	CAT	TAT	GAT	AGA	AAT	AAC	ATA	GCA	GTT	GGG	GCG	GAT	1824	
		Asp	Lys	Arg	Phe	His	Tyr	Asp	Arg	Asn	Asn	Ile	Ala	Val	Gly	Ala	Asp		
				595				600						605					
45		GAG	TCA	GTA	GTT	AAG	GAG	GCT	CAT	AGA	GAA	GTA	ATT	AAT	TCG	TCA	ACA	1872	
		Glu	Ser	Val	Val	Lys	Glu	Ala	His	Arg	Glu	Val	Ile	Asn	Ser	Ser	Thr		
			610					615					620						
50		GAG	GGA	TTA	TTG	TTA	AAT	ATT	GAT	AAG	GAT	ATA	AGA	AAA	ATA	TTA	TCA	1920	
		Glu	Gly	Leu	Leu	Leu	Asn	Ile	Asp	Lys	Asp	Ile	Arg	Lys	Ile	Leu	Ser		
			625				630					635					640		
55		GGT	TAT	ATT	GTA	GAA	ATT	GAA	GAT	ACT	GAA	GGG	CTT	AAA	GAA	GTT	ATA	1968	
		Gly	Tyr	Ile	Val	Glu	Ile	Glu	Asp	Thr	Glu	Gly	Leu	Lys	Glu	Val	Ile		
					645						650					655			
60		AAT	GAC	AGA	TAT	GAT	ATG	TTG	AAT	ATT	TCT	AGT	TTA	CGG	CAA	GAT	GGA	2016	
		Asn	Asp	Arg	Tyr	Asp	Met	Leu	Asn	Ile	Ser	Ser	Leu	Arg	Gln	Asp	Gly		
					660					665					670				
65		AAA	ACA	TTT	ATA	GAT	TIT	AAA	AAA	TAT	AAT	GAT	AAA	TTA	CCG	TTA	TAT	2064	
		Lys	Thr	Phe	Ile	Asp	Phe	Lys	Lys	Tyr	Asn	Asp	Lys	Leu	Pro	Leu	Tyr		
				675					680					685					
70		ATA	AGT	AAT	CCC	AAT	TAT	AAG	GTA	AAT	GTA	TAT	GCT	GTT	ACT	AAA	GAA	2112	
		Ile	Ser	Asn	Pro	Asn	Tyr	Lys	Val	Asn	Val	Tyr	Ala	Val	Thr	Lys	Glu		
			690					695					700						
75		AAC	ACT	ATT	ATT	AAT	CCT	ACT	GAG	AAT	GGG	GAT	ACT	AGT	ACC	AAC	GGG	2160	
		Asn	Thr	Ile	Ile	Asn	Pro	Ser	Glu	Asn	Gly	Asp	Thr	Ser	Thr	Asn	Gly		
			705				710					715					720		
80		ATC	AAG	AAA	ATT	TTA	AAG	AAA	GTG	GTG	CTG	GGC	AAA	AAA	GGG	GAT	ACA	2208	
		Ile	Lys	Lys	Ile	Leu	Lys	Lys	Val	Val	Leu	Gly	Lys	Lys	Gly	Asp	Thr		
					725						730					735			
85		GTG	GAA	CTG	ACC	TGT	ACA	GCT	TCC	CAG	AAG	AAG	AGC	ATA	CAA	TTC	CAC	2256	
		Val	Glu	Leu	Thr	Cys	Thr	Ala	Ser	Gln	Lys	Lys	Ser	Ile	Gln	Phe	His		

100

	740	745	750	
5	TGG AAA AAC TCC AAC CAG ATA AAG ATT CTG GGA AAT CAG GGC TCC TTC Trp Lys Asn Ser Asn Gln Ile Lys Ile Leu Gly Asn Gln Gly Ser Phe 755 760 765	2304		
10	TTA ACT AAA GGT CCA TCC AAG CTG AAT GAT CGC GCT GAC TCA AGA AGA Leu Thr Lys Gly Pro Ser Lys Leu Asn Asp Arg Ala Asp Ser Arg Arg 770 775 780	2352		
15	AGC CTT TGG GAC CAA GGA AAC TTC CCC CTG ATC ATC AAG AAT CTT AAG Ser Leu Trp Asp Gln Gly Asn Phe Pro Leu Ile Ile Lys Asn Leu Lys 785 790 795	2400		
20	ATA GAA GAC TCA GAT ACT TAC ATC TGT GAA GTG GAG GAC CAG AAG GAG Ile Glu Asp Ser Asp Thr Tyr Ile Cys Glu Val Glu Asp Gln Lys Glu 805 810 815	2448		
25	GAG GTG CAA TTG CTA GTG TTC GGA TTG ACT GCC AAC TCT GAC ACC CAC Glu Val Gln Leu Leu Val Phe Gly Leu Thr Ala Asn Ser Asp Thr His 820 825 830	2496		
30	CTG CTT CAG GGG CAG AGC CTG ACC CTG ACC TTG GAG AGC CCC CCT GGT Leu Leu Gln Gly Gln Ser Leu Thr Leu Thr Leu Glu Ser Pro Pro Gly 835 840 845	2544		
35	AGT AGC CCC TCA GTG CAA TGT AGG AGT CCA AGG GGT AAA AAC ATA CAG Ser Ser Pro Ser Val Gln Cys Arg Ser Pro Arg Gly Lys Asn Ile Gln 850 855 860	2592		
40	GGG GGG AAG ACC CTC TCC GTG TCT CAG CTG GAG CTC CAG GAT AGT GGC Gly Gly Lys Thr Leu Ser Val Ser Gln Leu Glu Leu Gln Asp Ser Gly 865 870 875 880	2640		
45	ACC TGG ACA TGC ACT GTC TTG CAG AAC CAG AAG AAG GTG GAG TTC AAA Thr Trp Thr Cys Thr Val Leu Gln Asn Gln Lys Lys Val Glu Phe Lys 885 890 895	2688		
50	ATA GAC ATC GTG GTG CTA GCT Ile Asp Ile Val Val Leu Ala 900	2709		

(2) INFORMATION FOR SEQ ID NO:12:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 903 amino acids

(B) TYPE: amino acid

(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: protein

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:12

55 Glu Val Lys Gln Glu Asn Arg Leu Leu Asn Glu Ser Glu Ser Ser Ser
1 5 10 15

101

Gln Gly Leu Leu Gly Tyr Tyr Phe Ser Asp Leu Asn Phe Gln Ala Pro
 20 25 30
 5 Met Val Val Thr Ser Ser Thr Thr Gly Asp Leu Ser Ile Pro Ser Ser
 35 40 45
 Glu Leu Glu Asn Ile Pro Ser Glu Asn Gln Tyr Phe Gln Ser Ala Ile
 50 55 60
 10 Trp Ser Gly Phe Ile Lys Val Lys Lys Ser Asp Glu Tyr Thr Phe Ala
 65 70 75 80
 Thr Ser Ala Asp Asn His Val Thr Met Trp Val Asp Asp Gln Glu Val
 85 90 95
 15 Ile Asn Lys Ala Ser Asn Ser Asn Lys Ile Arg Leu Glu Lys Gly Arg
 100 105 110
 Leu Tyr Gln Ile Lys Ile Gln Tyr Gln Arg Glu Asn Pro Thr Glu Lys
 115 120 125
 20 Gly Leu Asp Phe Lys Leu Tyr Trp Thr Asp Ser Gln Asn Lys Lys Glu
 130 135 140
 25 Val Ile Ser Ser Asp Asn Leu Gln Leu Pro Glu Leu Lys Gln Lys Ser
 145 150 155 160
 Ser Asn Ser Arg Lys Lys Arg Ser Thr Ser Ala Gly Pro Thr Val Pro
 165 170 175
 30 Asp Arg Asp Asn Asp Gly Ile Pro Asp Ser Leu Glu Val Glu Gly Tyr
 180 185 190
 35 Thr Val Asp Val Lys Asn Lys Arg Thr Phe Leu Ser Pro Trp Ile Ser
 195 200 205
 Asn Ile His Glu Lys Lys Gly Leu Thr Lys Tyr Lys Ser Ser Pro Glu
 210 215 220
 40 Lys Trp Ser Thr Ala Ser Asp Pro Tyr Ser Asp Phe Glu Lys Val Thr
 225 230 235 240
 Gly Arg Ile Asp Lys Asn Val Ser Pro Glu Ala Arg His Pro Leu Val
 245 250 255
 45 Ala Ala Tyr Pro Ile Val His Val Asp Met Glu Asn Ile Ile Leu Ser
 260 265 270
 50 Lys Asn Glu Asp Gln Ser Thr Gln Asn Thr Asp Ser Glu Thr Arg Thr
 275 280 285
 Ile Ser Lys Asn Thr Ser Thr Ser Arg Thr His Thr Ser Glu Val His
 290 295 300
 55 Gly Asn Ala Glu Val His Ala Ser Phe Phe Asp Ile Gly Gly Ser Val
 305 310 315 320
 Ser Ala Gly Phe Ser Asn Ser Asn Ser Ser Thr Val Ala Ile Asp His
 325 330 335
 60 Ser Leu Ser Leu Ala Gly Glu Arg Thr Trp Ala Glu Thr Met Gly Leu
 340 345 350
 Asn Thr Ala Asp Thr Ala Arg Leu Asn Ala Asn Ile Arg Tyr Val Asn
 355 360 365
 65 Thr Gly Thr Ala Pro Ile Tyr Asn Val Leu Pro Thr Thr Ser Leu Val
 370 375 380
 Leu Gly Lys Asn Gln Thr Leu Ala Thr Ile Lys Ala Lys Glu Asn Gln

102

	385		390		395		400
	Leu Ser Gln Ile	Leu Ala Pro Asn Asn Tyr Tyr Pro Ser Lys Asn Leu					
		405		410			415
5	Ala Pro Ile Ala Leu Asn Ala Gln Asp Asp Phe Ser Ser Thr Pro Ile						
		420		425			430
10	Thr Met Asn Tyr Asn Gln Phe Leu Glu Leu Glu Lys Thr Lys Gln Leu						
		435		440			445
	Arg Leu Asp Thr Asp Gln Val Tyr Gly Asn Ile Ala Thr Tyr Asn Phe						
		450		455			460
15	Glu Asn Gly Arg Val Arg Val Asp Thr Gly Ser Asn Trp Ser Glu Val						
		465		470			475
	Leu Pro Gln Ile Gln Glu Thr Thr Ala Arg Ile Ile Phe Asn Gly Lys						
			485		490		495
20	Asp Leu Asn Leu Val Glu Arg Arg Ile Ala Ala Val Asn Pro Ser Asp						
		500		505			510
25	Pro Leu Glu Thr Thr Lys Pro Asp Met Thr Leu Lys Glu Ala Leu Lys						
		515		520			525
	Ile Ala Phe Gly Phe Asn Glu Pro Asn Gly Asn Leu Gln Tyr Gln Gly						
		530		535			540
30	Lys Asp Ile Thr Glu Phe Asp Phe Asn Phe Asp Gln Gln Thr Ser Gln						
		545		550			555
	Asn Ile Lys Asn Gln Leu Ala Glu Leu Asn Ala Thr Asn Ile Tyr Thr						
			565		570		575
35	Val Leu Asp Lys Ile Lys Leu Asn Ala Lys Met Asn Ile Leu Ile Arg						
		580		585			590
40	Asp Lys Arg Phe His Tyr Asp Arg Asn Asn Ile Ala Val Gly Ala Asp						
		595		600			605
	Glu Ser Val Val Lys Glu Ala His Arg Glu Val Ile Asn Ser Ser Thr						
		610		615			620
45	Glu Gly Leu Leu Leu Asn Ile Asp Lys Asp Ile Arg Lys Ile Leu Ser						
		625		630			635
	Gly Tyr Ile Val Glu Ile Glu Asp Thr Glu Gly Leu Lys Glu Val Ile						
			645		650		655
50	Asn Asp Arg Tyr Asp Met Leu Asn Ile Ser Ser Leu Arg Gln Asp Gly						
		660		665			670
	Lys Thr Phe Ile Asp Phe Lys Lys Tyr Asn Asp Lys Leu Pro Leu Tyr						
		675		680			685
55	Ile Ser Asn Pro Asn Tyr Lys Val Asn Val Tyr Ala Val Thr Lys Glu						
		690		695			700
60	Asn Thr Ile Ile Asn Pro Ser Glu Asn Gly Asp Thr Ser Thr Asn Gly						
		705		710			715
	Ile Lys Lys Ile Leu Lys Lys Val Val Leu Gly Lys Lys Gly Asp Thr						
			725		730		735
65	Val Glu Leu Thr Cys Thr Ala Ser Gln Lys Lys Ser Ile Gln Phe His						
		740		745			750
	Trp Lys Asn Ser Asn Gln Ile Lys Ile Leu Gly Asn Gln Gly Ser Phe						
		755		760			765

[illegible]

(2) INFORMATION FOR SEQ ID NO:13:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 8 amino acids
- (B) TYPE: amino acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: peptide

(iii) HYPOTHETICAL: NO

(v) FRAGMENT TYPE: internal

(vi) ORIGINAL SOURCE:

- (A) ORGANISM: Bacillus anthracis

(ix) FEATURE:

- (A) NAME/KEY: Peptide
- (B) LOCATION: 1..8
- (D) OTHER INFORMATION: /label= PAHIV

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:13:

Ser Gln Asn Tyr Pro Val Val Gln
1 5

(2) INFORMATION FOR SEQ ID NO:14:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 12 amino acids
- (B) TYPE: amino acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: peptide

(iii) HYPOTHETICAL: NO

(v) FRAGMENT TYPE: internal

(vi) ORIGINAL SOURCE:

- (A) ORGANISM: Bacillus anthracis

(ix) FEATURE:

- (A) NAME/KEY: Peptide
- (B) LOCATION: 1..12
- (D) OTHER INFORMATION: /label= PAHIV-1

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:14:

Gln Val Ser Gln Asn Tyr Pro Ile Val Gln Asn Ile
1 5 10

(2) INFORMATION FOR SEQ ID NO:15:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 12 amino acids
- (B) TYPE: amino acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: peptide

(iii) HYPOTHETICAL: NO

(v) FRAGMENT TYPE: internal

105

(vi) ORIGINAL SOURCE:
(A) ORGANISM: Bacillus anthracis

(ix) FEATURE:
(A) NAME/KEY: Peptide
(B) LOCATION: 1..12
(D) OTHER INFORMATION: /label= PAHIV-2

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:15:

Asn Thr Ala Thr Ile Met Met Gln Arg Gly Asn Phe
1 5 10

(2) INFORMATION FOR SEQ ID NO:16:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 12 amino acids
(B) TYPE: amino acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: peptide

(iii) HYPOTHETICAL: NO

(v) FRAGMENT TYPE: internal

(vi) ORIGINAL SOURCE:
(A) ORGANISM: Bacillus anthracis

(ix) FEATURE:
(A) NAME/KEY: Peptide
(B) LOCATION: 1..12
(D) OTHER INFORMATION: /label= PAHIV-3

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:16:

Thr Val Ser Phe Asn Phe Pro Gln Ile Thr Leu Trp
1 5 10

(2) INFORMATION FOR SEQ ID NO:17:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 13 amino acids
(B) TYPE: amino acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: peptide

(iii) HYPOTHETICAL: NO

(v) FRAGMENT TYPE: internal

(vi) ORIGINAL SOURCE:
(A) ORGANISM: Bacillus anthracis

(ix) FEATURE:
(A) NAME/KEY: Peptide
(B) LOCATION: 1..13
(D) OTHER INFORMATION: /label= PAHIV-4

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:17:

Gly Gly Ser Ala Phe Asn Phe Pro Ile Val Met Gly Gly
1 5 10

106

(2) INFORMATION FOR SEQ ID NO:18:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 45 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(iii) HYPOTHETICAL: NO

(vi) ORIGINAL SOURCE:

- (A) ORGANISM: Bacillus anthracis

(ix) FEATURE:

- (A) NAME/KEY: CDS
(B) LOCATION: 3..44
(D) OTHER INFORMATION: /product= "Primer 1A"

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:18:

CG CAA GTA TCA CAA AAT TAT CCG ATC GTG CAA AAC ATA CTG CAG 44
Gln Val Ser Gln Asn Tyr Pro Ile Val Gln Asn Ile Leu Gln
1 5 10

G

(2) INFORMATION FOR SEQ ID NO:19:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 14 amino acids
(B) TYPE: amino acid
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: protein

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:19:

Gln Val Ser Gln Asn Tyr Pro Ile Val Gln Asn Ile Leu Gln
1 5 10

(2) INFORMATION FOR SEQ ID NO:20:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 46 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(iii) HYPOTHETICAL: NO

(iv) ANTI-SENSE: YES

(vi) ORIGINAL SOURCE:

- (A) ORGANISM: Bacillus anthracis

(ix) FEATURE:

- (A) NAME/KEY: misc feature
(B) LOCATION: 1..46
(D) OTHER INFORMATION: /product= "PRIMER 1B"

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:20:

GTTCTGTCAG TATGTTTTGC ACGATCGGAT AATTTTGTGA TACTTG

46

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(2) INFORMATION FOR SEQ ID NO:21:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 45 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(iii) HYPOTHETICAL: NO

(vi) ORIGINAL SOURCE:

- (A) ORGANISM: Bacillus anthracis

(ix) FEATURE:

- (A) NAME/KEY: CDS
- (B) LOCATION: 3..44
- (D) OTHER INFORMATION: /product= "Primer 2A"

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:21:

CG AAC ACT GCC ACT ATC ATG ATG CAA CGT GGT AAT TTT CTG CAG 44
Asn Thr Ala Thr Ile Met Met Gln Arg Gly Asn Phe Leu Gln
1 5 10

G 45

(2) INFORMATION FOR SEQ ID NO:22:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 14 amino acids
- (B) TYPE: amino acid
- (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: protein

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:22:

Asn Thr Ala Thr Ile Met Met Gln Arg Gly Asn Phe Leu Gln
1 5 10

(2) INFORMATION FOR SEQ ID NO:23:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 46 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(iii) HYPOTHETICAL: NO

(iv) ANTI-SENSE: YES

(vi) ORIGINAL SOURCE:

- (A) ORGANISM: Bacillus anthracis

(ix) FEATURE:

- (A) NAME/KEY: misc_feature
- (B) LOCATION: 1..46
- (D) OTHER INFORMATION: /product= "PRIMER 2B"

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:23:

GTCCCTGCAG AAAATTACCA CGTTGCATCA TGATAGTGGC AGTGTT

46

(2) INFORMATION FOR SEQ ID NO:24:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 45 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(iii) HYPOTHETICAL: NO

(vi) ORIGINAL SOURCE:

- (A) ORGANISM: Bacillus anthracis

(ix) FEATURE:

- (A) NAME/KEY: CDS
- (B) LOCATION: 3..44
- (D) OTHER INFORMATION: /product= "Primer 3A"

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:24:

CG ACT GTC TCT TTT AAC TTC CCG CAA ATC ACG CTT TGG CTG CAG
Thr Val Ser Phe Asn Phe Pro Gln Ile Thr Leu Trp Leu Gln
1 5 10

44

G

45

(2) INFORMATION FOR SEQ ID NO:25:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 14 amino acids
- (B) TYPE: amino acid
- (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: protein

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:25:

Thr Val Ser Phe Asn Phe Pro Gln Ile Thr Leu Trp Leu Gln
1 5 10

(2) INFORMATION FOR SEQ ID NO:26:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 46 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(iii) HYPOTHETICAL: NC

(iv) ANTI-SENSE: YES

(vi) ORIGINAL SOURCE:

- (A) ORGANISM: Bacillus anthracis

(ix) FEATURE:

- (A) NAME/KEY: misc_feature
- (B) LOCATION: 1..46
- (D) OTHER INFORMATION: /product= "PRIMER 3B"

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:26:

GTCCCTGCAG CCAAAGCGTG ATTTGCGGGA AGTTAAAAGA GACAGT

46

5 (2) INFORMATION FOR SEQ ID NO:27:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 48 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

10

(ii) MOLECULE TYPE: DNA (genomic)

15

(iii) HYPOTHETICAL: NO

(vi) ORIGINAL SOURCE:

(A) ORGANISM: Bacillus anthracis

20

(ix) FEATURE:

- (A) NAME/KEY: CDS
(B) LOCATION: 3..47
(D) OTHER INFORMATION: /product= "Primer 4A"

25

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:27:

CG GGC GGT TCT GCC TTT AAC TTC CCG ATC GTC ATG GGA GGT CTG CAG
Gly Gly Ser Ala Phe Asn Phe Pro Ile Val Met Gly Gly Leu Gln
1 5 10 15

30

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47

48

35 (2) INFORMATION FOR SEQ ID NO:28:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 15 amino acids
(B) TYPE: amino acid
(D) TOPOLOGY: linear

40

(ii) MOLECULE TYPE: protein

110

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:28:

Gly Gly Ser Ala Phe Asn Phe Pro Ile Val Met Gly Gly Leu Gln
1 5 10 15

5

(2) INFORMATION FOR SEQ ID NO:29:

(i) SEQUENCE CHARACTERISTICS:

10

- (A) LENGTH: 49 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

15

(ii) MOLECULE TYPE: DNA (genomic)

(iii) HYPOTHETICAL: NO

(iv) ANTI-SENSE: YES

20

(vi) ORIGINAL SOURCE:

(A) ORGANISM: Bacillus anthracis

(ix) FEATURE:

25

- (A) NAME/KEY: misc_feature
- (B) LOCATION: 1..49
- (D) OTHER INFORMATION: /product= "PRIMER 4B"

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:29:

30

GTCCCTGCAG ACCTCCCATG ACGATCGGGA AGTTAAAGGC AGAACCGCC

49

(2) INFORMATION FOR SEQ ID NO:30:

35

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 2160 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

40

(ii) MOLECULE TYPE: DNA (genomic)

(iii) HYPOTHETICAL: NO

45

(vi) ORIGINAL SOURCE:

(A) ORGANISM: Bacillus anthracis

(ix) FEATURE:

50

- (A) NAME/KEY: CDS
- (B) LOCATION: 1..2157
- (D) OTHER INFORMATION: /product= "PAHIV#2"

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(xi) SEQUENCE DESCRIPTION: SEQ ID NO:30:

5	GAA GTT AAA CAG GAG AAC CGG TTA TTA AAT GAA TCA GAA TCA AGT TCC Glu Val Lys Gln Glu Asn Arg Leu Leu Asn Glu Ser Glu Ser Ser Ser	48
10	CAG GGG TTA CTA GGA TAC TAT TTT AGT GAT TTG AAT TTT CAA GCA CCC Gln Gly Leu Leu Gly Tyr Tyr Phe Ser Asp Leu Asn Phe Gln Ala Pro	96
15	ATG GTG GTT ACC TCT TCT ACT ACA GGG GAT TTA TCT ATT CCT AGT TCT Met Val Val Thr Ser Ser Thr Thr Gly Asp Leu Ser Ile Pro Ser Ser	144
20	GAG TTA GAA AAT ATT CCA TCG GAA AAC CAA TAT TTT CAA TCT GCT ATT Glu Leu Glu Asn Ile Pro Ser Glu Asn Gln Tyr Phe Gln Ser Ala Ile	192
25	TGG TCA GGA TTT ATC AAA GTT AAG AAG AGT GAT GAA TAT ACA TTT GCT Trp Ser Gly Phe Ile Lys Val Lys Lys Ser Asp Glu Tyr Thr Phe Ala	240
30	ACT TCC GCT GAT AAT CAT GTA ACA ATG TGG GTA GAT GAC CAA GAA GTG Thr Ser Ala Asp Asn His Val Thr Met Trp Val Asp Asp Gln Glu Val	288
35	ATT AAT AAA GCT TCT AAT TCT AAC AAA ATC AGA TTA GAA AAA GGA AGA Ile Asn Lys Ala Ser Asn Ser Asn Lys Ile Arg Leu Glu Lys Gly Arg	336
40	TTA TAT CAA ATA AAA ATT CAA TAT CAA CGA GAA AAT CCT ACT GAA AAA Leu Tyr Gln Ile Lys Ile Gln Tyr Gln Arg Glu Asn Pro Thr Glu Lys	384
45	GGA TTG GAT TTC AAG TTG TAC TGG ACC GAT TCT CAA AAT AAA AAA GAA Gly Leu Asp Phe Lys Leu Tyr Trp Thr Asp Ser Gln Asn Lys Lys Glu	432
50	GTG ATT TCT AGT GAT AAC TTA CAA TTG CCA GAA TTA AAA CAA AAA TCT Val Ile Ser Ser Asp Asn Leu Gln Leu Pro Glu Leu Lys Gln Lys Ser	480
55	TCG AAC ACT GCC ACT ATC ATG ATG CAA CGT GGT AAT TTT CTG CAG GGA Ser Asn Thr Ala Thr Ile Met Met Gln Arg Gly Asn Phe Leu Gln Gly	528
	CCT ACG GTT CCA GAC CGT GAC AAT GAT GGA ATC CCT GAT TCA TTA GAG Pro Thr Val Pro Asp Arg Asp Asn Asp Gly Ile Pro Asp Ser Leu Glu	576
	GTA GAA GGA TAT ACG GTT GAT GTC AAA AAT AAA AGA ACT TTT CTT TCA Val Glu Gly Tyr Thr Val Asp Val Lys Asn Lys Arg Thr Phe Leu Ser	624

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	CCA TGG ATT TCT AAT ATT CAT GAA AAG AAA GGA TTA ACC AAA TAT AAA	672
	Pro Trp Ile Ser Asn Ile His Glu Lys Lys Gly Leu Thr Lys Tyr Lys	
	210 215 220	
5	TCA TCT CCT GAA AAA TGG AGC ACG GCT TCT GAT CCG TAC AGT GAT TTC	720
	Ser Ser Pro Glu Lys Trp Ser Thr Ala Ser Asp Pro Tyr Ser Asp Phe	
	225 230 235 240	
10	GAA AAG GTT ACA GGA CGG ATT GAT AAG AAT GTA TCA CCA GAG GCA AGA	768
	Glu Lys Val Thr Gly Arg Ile Asp Lys Asn Val Ser Pro Glu Ala Arg	
	245 250 255	
15	CAC CCC CTT GTG GCA GCT TAT CCG ATT GTA CAT GTA GAT ATG GAG AAT	816
	His Pro Leu Val Ala Ala Tyr Pro Ile Val His Val Asp Met Glu Asn	
	260 265 270	
20	ATT ATT CTC TCA AAA AAT GAG GAT CAA TCC ACA CAG AAT ACT GAT AGT	864
	Ile Ile Leu Ser Lys Asn Glu Asp Gln Ser Thr Gln Asn Thr Asp Ser	
	275 280 285	
25	GAA ACG AGA ACA ATA AGT AAA AAT ACT TCT ACA AGT AGG ACA CAT ACT	912
	Glu Thr Arg Thr Ile Ser Lys Asn Thr Ser Thr Ser Arg Thr His Thr	
	290 295 300	
30	AGT GAA GTA CAT GGA AAT GCA GAA GTG CAT GCG TCG TTC TTT GAT ATT	960
	Ser Glu Val His Gly Asn Ala Glu Val His Ala Ser Phe Phe Asp Ile	
	305 310 315 320	
35	GGT GGG AGT GTA TCT GCA GGA TTT AGT AAT TCG AAT TCA AGT ACG GTC	1008
	Gly Gly Ser Val Ser Ala Gly Phe Ser Asn Ser Asn Ser Ser Thr Val	
	325 330 335	
40	GCA ATT GAT CAT TCA CTA TCT CTA GCA GGG GAA AGA ACT TGG GCT GAA	1056
	Ala Ile Asp His Ser Leu Ser Leu Ala Gly Glu Arg Thr Trp Ala Glu	
	340 345 350	
45	ACA ATG GGT TTA AAT ACC GCT GAT ACA GCA AGA TTA AAT GCC AAT ATT	1104
	Thr Met Gly Leu Asn Thr Ala Asp Thr Ala Arg Leu Asn Ala Asn Ile	
	355 360 365	
50	AGA TAT GTA AAT ACT GGG ACG GCT CCA ATC TAC AAC GTG TTA CCA ACG	1152
	Arg Tyr Val Asn Thr Gly Thr Ala Pro Ile Tyr Asn Val Leu Pro Thr	
	370 375 380	
55	ACT TCG TTA GTG TTA GGA AAA AAT CAA ACA CTC GCG ACA ATT AAA GCT	1200
	Thr Ser Leu Val Leu Gly Lys Asn Gln Thr Leu Ala Thr Ile Lys Ala	
	385 390 395 400	
60	AAG GAA AAC CAA TTA AGT CAA ATA CTT GCA CCT AAT AAT TAT TAT CCT	1248
	Lys Glu Asn Gln Leu Ser Gln Ile Leu Ala Pro Asn Asn Tyr Tyr Pro	
	405 410 415	
65	TCT AAA AAC TTG GCG CCA ATC GCA TTA AAT GCA CAA GAC GAT TTC AGT	1296
	Ser Lys Asn Leu Ala Pro Ile Ala Leu Asn Ala Gln Asp Asp Phe Ser	
	420 425 430	
70	TCT ACT CCA ATT ACA ATG AAT TAC GGG AAT ATA GCA ACA TAC AAT TTT	1344
	Ser Thr Pro Ile Thr Met Asn Tyr Gly Asn Ile Ala Thr Tyr Asn Phe	
	435 440 445	
75	GAA AAT GCA AGA GTG AGG GTG GAT ACA GGC TCG AAC TCG AGT GAA GTG	1392
	Glu Asn Gly Arg Val Arg Val Asp Thr Gly Ser Asn Trp Ser Glu Val	
	450 455 460	
80	TTA CCG CAA ATT CAA GAA ACA ACT GCA CGT ATC ATT TTT AAT GGA AAT	1440
	Leu Pro Gln Ile Gln Glu Thr Thr Ala Arg Ile Ile Phe Asn Gly Lys	
	465 470 475 480	
85	GAT TTA AAT CTG GTA GAA AGG CGG ATA GCG GCG GTT AAT CCT AGT GAT	1488

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	Asp	Leu	Asn	Leu	Val	Glu	Arg	Arg	Ile	Ala	Ala	Val	Asn	Pro	Ser	Asp	
				485						490					495		
5	CCA	TTA	GAA	ACG	ACT	AAA	CCG	GAT	ATG	ACA	TTA	AAA	GAA	GCC	CTT	AAA	1536
	Pro	Leu	Glu	Thr	Thr	Lys	Pro	Asp	Met	Thr	Leu	Lys	Glu	Ala	Leu	Lys	
				500					505					510			
10	ATA	GCA	TTT	GGA	TTT	AAC	GAA	CCG	AAT	GGA	AAC	TTA	CAA	TAT	CAA	GGG	1584
	Ile	Ala	Phe	Gly	Phe	Asn	Glu	Pro	Asn	Gly	Asn	Leu	Gln	Tyr	Gln	Gly	
			515					520					525				
15	AAA	GAC	ATA	ACC	GAA	TTT	GAT	TTT	AAT	TTC	GAT	CAA	CAA	ACA	TCT	CAA	1632
	Lys	Asp	Ile	Thr	Glu	Phe	Asp	Phe	Asn	Phe	Asp	Gln	Gln	Thr	Ser	Gln	
		530					535					540					
20	AAT	ATC	AAG	AAT	CAG	TTA	GCG	GAA	TTA	AAC	GCA	ACT	AAC	ATA	TAT	ACT	1680
	Asn	Ile	Lys	Asn	Gln	Ala	Glu	Leu	Asn	Ala	Thr	Asn	Ile	Tyr	Thr		
		545			550					555					560		
25	GTA	TTA	GAT	AAA	ATC	AAA	TTA	AAT	GCA	AAA	ATG	AAT	ATT	TTA	ATA	AGA	1728
	Val	Leu	Asp	Lys	Ile	Lys	Leu	Asn	Ala	Lys	Met	Asn	Ile	Leu	Ile	Arg	
					565					570					575		
30	GAT	AAA	CGT	TTT	CAT	TAT	GAT	AGA	AAT	AAC	ATA	GCA	GTT	GGG	GCG	GAT	1776
	Asp	Lys	Arg	Phe	His	Tyr	Asp	Arg	Asn	Asn	Ile	Ala	Val	Gly	Ala	Asp	
				580				585						590			
35	GAG	TCA	GTA	GTT	AAG	GAG	GCT	CAT	AGA	GAA	GTA	ATT	AAT	TCG	TCA	ACA	1824
	Glu	Ser	Val	Val	Lys	Glu	Ala	His	Arg	Glu	Val	Ile	Asn	Ser	Ser	Thr	
			595					600					605				
40	GAG	GGA	TTA	TTG	TTA	AAT	ATT	GAT	AAG	GAT	ATA	AGA	AAA	ATA	TTA	TCA	1872
	Glu	Gly	Leu	Leu	Leu	Asn	Ile	Asp	Lys	Asp	Ile	Arg	Lys	Ile	Leu	Ser	
		610					615					620					
45	GGT	TAT	ATT	GTA	GAA	ATT	GAA	GAT	ACT	GAA	GGG	CTT	AAA	GAA	GTT	ATA	1920
	Gly	Tyr	Ile	Val	Glu	Ile	Glu	Asp	Thr	Glu	Gly	Leu	Lys	Glu	Val	Ile	
						630				635					640		
50	AAT	GAC	AGA	TAT	GAT	ATG	TTG	AAT	ATT	TCT	AGT	TTA	CGG	CAA	GAT	GGA	1968
	Asn	Asp	Arg	Tyr	Asp	Met	Leu	Asn	Ile	Ser	Ser	Leu	Arg	Gln	Asp	Gly	
					645					650					655		
55	AAA	ACA	TTT	ATA	GAT	TTT	AAA	AAA	TAT	AAT	GAT	AAA	TTA	CCG	TTA	TAT	2016
	Lys	Thr	Phe	Ile	Asp	Phe	Lys	Lys	Tyr	Asn	Asp	Lys	Leu	Pro	Leu	Tyr	
				660					665					670			
60	ATA	AGT	AAT	CCC	AAT	TAT	AAG	GTA	AAT	GTA	TAT	GCT	GTT	ACT	AAA	GAA	2064
	Ile	Ser	Asn	Pro	Asn	Tyr	Lys	Val	Asn	Val	Tyr	Ala	Val	Thr	Lys	Glu	
			675					680					685				
65	AAC	ACT	ATT	ATT	AAT	CCT	AGT	GAG	AAT	GGG	GAT	ACT	AGT	ACC	AAC	GGG	2112
	Asn	Thr	Ile	Ile	Asn	Pro	Ser	Glu	Asn	Gly	Asp	Thr	Ser	Tor	Asn	Gly	
		690					695					700					
70	ATC	AAG	AAA	ATT	TTA	ATC	TTT	TCT	AAA	AAA	GGC	TAT	GAG	ATA	GGA		2157
	Ile	Lys	Lys	Ile	Leu	Ile	Phe	Ser	Lys	Lys	Gly	Tyr	Glu	Ile	Gly		
		705				710					715						
75	TAA																2160

(2) INFORMATION FOR SEQ ID NO:31:

- 65 (i) SEQUENCE CHARACTERISTICS:
- (A) LENGTH: 719 amino acids
 - (B) TYPE: amino acid
 - (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: protein

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:31:

5	Glu 1	Val	Lys	Gln	Glu 5	Asn	Arg	Leu	Leu	Asn 10	Glu	Ser	Glu	Ser	Ser	Ser	15
	Gln	Gly	Leu	Leu 20	Gly	Tyr	Tyr	Phe	Ser 25	Asp	Leu	Asn	Phe	Gln	Ala	Pro	30
10	Met	Val	Val	Thr	Ser	Ser	Thr	Thr	Gly	Asp	Leu	Ser	Ile	Pro	Ser	Ser	45
	Glu	Leu	Glu	Asn	Ile	Pro	Ser	Glu	Asn	Gln	Tyr	Phe	Gln	Ser	Ala	Ile	60
15	Trp 65	Ser	Gly	Phe	Ile	Lys 70	Val	Lys	Lys	Ser	Asp 75	Glu	Tyr	Thr	Phe	Ala	80
20	Thr	Ser	Ala	Asp	Asn 85	His	Val	Thr	Met	Trp 90	Val	Asp	Asp	Gln	Glu	Val	95
	Ile	Asn	Lys	Ala	Ser	Asn	Ser	Asn	Lys 105	Ile	Arg	Leu	Glu	Lys	Gly	Arg	110
25	Leu	Tyr	Gln	Ile	Lys	Ile	Gln	Tyr	Gln	Arg	Glu	Asn	Pro	Thr	Glu	Lys	125
	Gly	Leu	Asp	Phe	Lys	Leu	Tyr	Trp	Thr	Asp	Ser	Gln	Asn	Lys	Lys	Glu	140
30	Val 145	Ile	Ser	Ser	Asp	Asn 150	Leu	Gln	Leu	Pro	Glu	Leu	Lys	Gln	Lys	Ser	160
	Ser	Asn	Thr	Ala	Thr	Ile	Met	Met	Gln	Arg	Gly	Asn	Phe	Leu	Gln	Gly	175
35	Pro	Thr	Val	Pro	Asp	Arg	Asp	Asn	Asp	Gly	Ile	Pro	Asp	Ser	Leu	Glu	190
	Val	Glu	Gly	Tyr	Thr	Val	Asp	Val	Lys	Asn	Lys	Arg	Thr	Phe	Leu	Ser	205
40	Pro	Trp	Ile	Ser	Asn	Ile	His	Glu	Lys	Lys	Gly	Leu	Thr	Lys	Tyr	Lys	220
45	Ser 225	Ser	Pro	Glu	Lys	Trp 230	Ser	Thr	Ala	Ser	Asp 235	Pro	Tyr	Ser	Asp	Phe	240
	Glu	Lys	Val	Thr	Gly	Arg	Ile	Asp	Lys	Asn	Val	Ser	Pro	Glu	Ala	Arg	255
50	His	Pro	Leu	Val	Ala	Ala	Tyr	Pro	Ile	Val	His	Val	Asp	Met	Glu	Asn	270
	Ile	Ile	Leu	Ser	Lys	Asn	Glu	Asp	Gln	Ser	Thr	Gln	Asn	Thr	Asp	Ser	285
55	Glu	Thr	Arg	Thr	Ile	Ser	Lys	Asn	Thr	Ser	Thr	Ser	Arg	Thr	His	Thr	300
60	Ser 305	Glu	Val	His	Gly	Asn 310	Ala	Glu	Val	His	Ala 315	Ser	Phe	Phe	Asp	Ile	320
	Gly	Gly	Ser	Val	Ser	Ala	Gly	Phe	Ser	Asn	Ser	Asn	Ser	Ser	Thr	Val	335
65	Ala	Ile	Asp	His	Ser	Leu	Ser	Leu	Ala	Gly	Glu	Arg	Thr	Trp	Ala	Glu	350

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	Thr	Met	Gly	Leu	Asn	Thr	Ala	Asp	Thr	Ala	Arg	Leu	Asn	Ala	Asn	Ile	
			355					360					365				
5	Arg	Tyr	Val	Asn	Thr	Gly	Thr	Ala	Pro	Ile	Tyr	Asn	Val	Leu	Pro	Thr	
		370					375					380					
	Thr	Ser	Leu	Val	Leu	Gly	Lys	Asn	Gln	Thr	Leu	Ala	Thr	Ile	Lys	Ala	
	385					390					395					400	
10	Lys	Glu	Asn	Gln	Leu	Ser	Gln	Ile	Leu	Ala	Pro	Asn	Asn	Tyr	Tyr	Pro	
					405					410					415		
	Ser	Lys	Asn	Leu	Ala	Pro	Ile	Ala	Leu	Asn	Ala	Gln	Asp	Asp	Phe	Ser	
15				420					425					430			
	Ser	Thr	Pro	Ile	Thr	Met	Asn	Tyr	Gly	Asn	Ile	Ala	Thr	Tyr	Asn	Phe	
			435					440					445				
	Glu	Asn	Gly	Arg	Val	Arg	Val	Asp	Thr	Gly	Ser	Asn	Trp	Ser	Glu	Val	
20			450				455					460					
	Leu	Pro	Gln	Ile	Gln	Glu	Thr	Thr	Ala	Arg	Ile	Ile	Phe	Asn	Gly	Lys	
	465					470					475					480	
	Asp	Leu	Asn	Leu	Val	Glu	Arg	Arg	Ile	Ala	Ala	Val	Asn	Pro	Ser	Asp	
25				485						490					495		
	Pro	Leu	Glu	Thr	Thr	Lys	Pro	Asp	Met	Thr	Leu	Lys	Glu	Ala	Leu	Lys	
				500					505					510			
30	Ile	Ala	Phe	Gly	Phe	Asn	Glu	Pro	Asn	Gly	Asn	Leu	Gln	Tyr	Gln	Gly	
			515					520					525				
	Lys	Asp	Ile	Thr	Glu	Phe	Asp	Phe	Asn	Phe	Asp	Gln	Gln	Thr	Ser	Gln	
35		530					535					540					
	Asn	Ile	Lys	Asn	Gln	Leu	Ala	Glu	Leu	Asn	Ala	Thr	Asn	Ile	Tyr	Thr	
	545					550					555					560	
	Val	Leu	Asp	Lys	Ile	Lys	Leu	Asn	Ala	Lys	Met	Asn	Ile	Leu	Ile	Arg	
40				565						570					575		
	Asp	Lys	Arg	Phe	His	Tyr	Asp	Arg	Asn	Asn	Ile	Ala	Val	Gly	Ala	Asp	
			580					585						590			
45	Glu	Ser	Val	Val	Lys	Glu	Ala	His	Arg	Glu	Val	Ile	Asn	Ser	Ser	Thr	
			595					600					605				
	Glu	Gly	Leu	Leu	Leu	Asn	Ile	Asp	Lys	Asp	Ile	Arg	Lys	Ile	Leu	Ser	
50		610					615					620					
	Gly	Tyr	Ile	Val	Glu	Ile	Glu	Asp	Thr	Glu	Gly	Leu	Lys	Glu	Val	Ile	
	625					630					635					640	
	Asn	Asp	Arg	Tyr	Asp	Met	Leu	Asn	Ile	Ser	Ser	Leu	Arg	Gln	Asp	Gly	
55				645						650					655		
	Lys	Thr	Phe	Ile	Asp	Phe	Lys	Lys	Tyr	Asn	Asp	Lys	Leu	Pro	Leu	Tyr	
			660						665					670			
60	Ile	Ser	Asn	Pro	Asn	Tyr	Lys	Val	Asn	Val	Tyr	Ala	Val	Thr	Lys	Glu	
			675					680					685				
	Asn	Thr	Ile	Ile	Asn	Pro	Ser	Glu	Asn	Gly	Asp	Thr	Ser	Thr	Asn	Gly	
65			690				695					700					
	Ile	Lys	Lys	Ile	Leu	Ile	Phe	Ser	Lys	Lys	Gly	Tyr	Glu	Ile	Gly		
	705					710					715						

WHAT IS CLAIMED IS:

1. A nucleic acid encoding a fusion protein,
comprising a nucleotide sequence encoding the anthrax
5 protective antigen (PA) binding domain of the native anthrax
lethal factor (LF) protein and a nucleotide sequence encoding
an activity inducing domain of a second protein.
2. The nucleic acid of claim 1, wherein the second
10 protein is a toxin.
3. The nucleic acid of claim 2, wherein the toxin is
Pseudomonas exotoxin A.
4. The nucleic acid of claim 2, wherein the toxin is
15 the A chain of *Diphtheria* toxin.
5. The nucleic acid of claim 2, wherein the toxin is
shiga toxin.
20
6. The nucleic acid of claim 1, comprising the
nucleotide sequence defined in the Sequence Listing as SEQ ID
NO:5.
7. The nucleic acid of claim 1, comprising the
25 nucleotide sequence defined in the Sequence Listing as SEQ ID
NO:6.
8. A protein encoded by the nucleic acid of claim 1.
30
9. A vector comprising the nucleic acid of claim 1.
10. The vector of claim 9 in a host capable of
expressing the protein encoded by the nucleic acid.
35
11. A nucleic acid encoding a fusion protein, the
nucleic acid comprising a nucleotide sequence encoding the
translocation domain and anthrax lethal factor (LF) binding

domain of native anthrax protective antigen (PA) protein and a nucleotide sequence encoding a ligand domain which specifically binds a cellular target.

5 12. The nucleic acid of claim 11, wherein the ligand domain specifically binds to an HIV protein expressed on the surface of an HIV-infected cell.

10 13. The nucleic acid of claim 11, wherein the ligand domain is a growth factor.

15 14. The nucleic acid of claim 11, wherein the nucleotide sequence encoding the translocation domain and LF binding domain of the native PA protein further comprises the nucleotide sequence encoding the remainder of the native PA protein.

20 15. A protein encoded by the nucleic acid of claim 11.

16. A vector comprising the nucleic acid of claim 11.

25 17. The vector of claim 16 in a host capable of expressing the protein encoded by the nucleic acid.

18. A method of killing a tumor cell in a subject, the method comprising the steps of:

30 a) administering to the subject a first fusion protein comprising the translocation domain and LF binding domain of the native PA protein and a tumor cell specific ligand domain in an amount sufficient to bind to a tumor cell; and

35 b) administering to the subject a second fusion protein comprising the PA binding domain of the native LF protein and a cytotoxic domain of a non-LF protein in an amount sufficient to bind to the first protein, whereby the second protein is internalized into the tumor cell and kills the tumor cell.

19. A method of killing HIV-infected cells in a subject, the method comprising the steps of:

- a) administering to the subject a first fusion protein comprising the translocation domain and LF binding domain of the native PA protein and a ligand domain that specifically binds to an HIV protein expressed on the surface of an HIV-infected cell in an amount sufficient to bind to an HIV-infected cell; and
- b) administering to the subject a second fusion protein comprising the PA binding domain of the native LF protein and a cytotoxic domain of a non-LF protein in an amount sufficient to bind to the first protein, whereby the second protein is internalized into the HIV-infected cell and kills the HIV-infected cell, thereby preventing propagation of HIV.

20. A method for delivering an activity to a cell comprising the steps of:

- a) administering to the cell a protein comprising the translocation domain and the LF binding domain of the native PA protein and a ligand domain; and
- b) administering to the cell a compound comprising the PA binding domain of the native LF protein chemically attached to an activity inducing moiety, whereby the compound administered in step b) is internalized into the cell and effects the activity within the cell.

21. The method of claim 20, wherein the ligand domain is the receptor binding domain of the native PA protein.

22. The method of claim 20, wherein the activity inducing moiety is a polypeptide.

23. The method of claim 22, wherein the polypeptide is a growth factor.

24. The method of claim 20, wherein the activity inducing moiety is an antisense nucleic acid.

25. The method of claim 20, wherein the activity inducing moiety is a nucleic acid encoding a desired gene product.

5 26. A compound comprising the PA binding domain of the native LF protein chemically attached to a non-LF activity inducing moiety.

10 27. The composition of claim 26, wherein the activity inducing moiety is a polypeptide.

28. The composition of claim 26, wherein the activity inducing moiety is a radioisotope.

15 29. The composition of claim 26, wherein the activity inducing moiety is an antisense nucleic acid.

20 30. The composition of claim 26, wherein the activity inducing moiety is a nucleic acid encoding a desired gene product.

25 31. The nucleic acid of claim 11, comprising the nucleotide sequence defined in the Sequence Listing as SEQ ID NO:11.

32. A nucleic acid comprising a nucleotide sequence encoding an anthrax protective antigen which is altered to include a cleavage site recognized by a protease produced by an intracellular pathogen.

30 33. The nucleic acid of claim 32 wherein the intracellular pathogen is a virus.

35 34. The nucleic acid of claim 33 wherein the alteration comprises a mutation in at least one of amino acid residues 164-167 (the trypsin cleavage site).

35. The nucleic acid of claim 34 wherein the virus is a retrovirus.

5 36. The nucleic acid of claim 35 wherein the retrovirus is an HIV.

37. The nucleic acid of claim 36 wherein the amino acids at residues 164-167 are replaced with an amino acid sequence selected from the group comprising NTATIMMQRGNF, 10 QVSQNYPIVQNI, TVSFNFPQITLW, and GGSAFNFPIVMGG.

38. A polypeptide comprising an amino acid sequence encoding an anthrax protective antigen which is altered to include a cleavage site recognized by a protease produced by a 15 retrovirus.

39. The polypeptide of claim 38 wherein the alteration comprises a mutation in at least one of amino acid residues 164-167 (the trypsin cleavage site).

20 40. The polypeptide of claim 39 wherein the retrovirus is an HIV.

41. The polypeptide of claim 40 wherein the amino acid residues 164-167 are replaced with an amino acid sequence selected from the group comprising NTATIMMQRGNF, QVSQNYPIVQNI, 25 TVSFNFPQITLW, and GGSAFNFPIVMGG.

42. A method of killing a cell which is infected with an intracellular pathogen, the method comprising:

30 applying to the cell a composition comprising an effective amount an altered anthrax protective antigen (PA) having a cleavage site recognized by a protease produced by the intracellular pathogen.

35 43. The method of claim 42 wherein the cleavage site is at amino acid residues 164-167.

44. The method of claim 42 wherein the intracellular pathogen is a virus.

5 45. The method of claim 44 wherein the virus is a retrovirus.

46. A method of claim 45 wherein the retrovirus is an HIV.

10 47. The method of claim 46 wherein the amino acids at residues 164-167 are replaced with an amino acid sequence selected from the group comprising NTATIMMQRGNF, QVSQNYPIVQNI, TVSFNFPQITLW, and GGSANFPPIVMGG.

15 48. The method of claim 42 wherein the cell is harbored in a human.

20 49. The method of claim 48 wherein the step of applying the composition includes parenterally administering the composition to the human.

50. The method of claim 49 wherein the parenteral administration is intravenous.

25 51. The method of claim 48 wherein the effective amount of altered protective antigen is from about 5 to about 25 micrograms per kilogram of body weight of a human harboring the infected cell.

30 52. The method of claim 51 wherein the effective amount of altered protective antigen is about 10 micrograms per kilogram of body weight of a human harboring the infected cell.

Figure 1

Cleavage of mutant PAHIV proteins with purified HIV-1 protease

